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The complex jigsaw of HIV, medication, inflammation and coagulation

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The work presented in this thesis was carried out within the Radboud Institute for Health Sciences. The research was performed at the Department of Internal Medicine and Radboud Center for Infectious Diseases of the Radboud university medical center, Nijmegen, The Netherlands.

Financial support was provided by Radboud university medical center, Dutch Aidsfonds and ViiV Healthcare.

Printing of this thesis was financially supported by the Radboud University.

ISBN: 978-94-93270-03-9

Cover: Guus Gijben | Proefschrift-aio.nl

Lay-out: Guus Gijben | Proefschrift-aio.nl

Printing: Proefschrift-aio.nl

The complex jigsaw of HIV, medication, inflammation and coagulation.

Proefschrift

ter verkrijging van de graad van doctor
aan de Radboud Universiteit Nijmegen
op gezag van de rector magnificus prof. dr. J.H.J.M. van Krieken,
volgens besluit van het college van decanen
in het openbaar te verdedigen op woensdag 29 september 2021
om 12:30 uur precies

door

Wouter Anton van der Heijden
geboren op 15 oktober 1987
te Utrecht

Promotores:

Prof. dr. A.J.A.M van der Ven

Prof. dr. M.G. Netea

Co-promotor:

dr. Q. de Mast

Manuscriptcommissie

Prof. dr. D.M. Burger

Prof. dr. P. Pickkers

Prof. dr. A. Verbon (Universitair Medisch Centrum Utrecht)

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Chapter 1

General introduction

Human immunodeficiency virus 1 (HIV-1) was recognized as the causal agent of the acquired immune deficiency syndrome (AIDS) in 1983[1,2]. HIV infects cells that express the cluster differentiation (CD)4 receptor, including T cells (CD4+ T-cells)[3], monocytes/macrophages[4], and dendritic cells[5]. C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4) are important co-receptors that HIV uses to infect cells expressing CD4[6-8].

An acute HIV infection can be seen 2-4 weeks after exposure and mostly presents as a 'flu-like' syndrome although some individuals experience no significant symptoms. This acute HIV infection is followed by clinical latency or chronic HIV infection for 3-20 years[9,10]. Without treatment, the natural course of HIV infection causes progressive depletion of $CD4^+$ T cells, leading to severe immune suppression and subsequent development of opportunistic infections and viral induced cancers: a condition that was named AIDS in eighties[11]. AIDS was associated with a very poor prognosis, as the initial life expectancy of AIDS was reported to be between 6-12 months[11].

Antiretroviral therapy

In 1987, the Federal Drug Authority (FDA) in the United states approved zidovudine (AZT), a nucleoside reverse transcriptase inhibitor (NRTI), as the first antiretroviral drug to treat HIV[12]. The initial use as monotherapy resulted in rapid development of resistance, hampering long-term successful treatment. The discovery of other classes of antiretroviral drugs such as protease inhibitors[13,14] (PI) and nonnucleoside reverse-transcriptase inhibitors[15] (NNRTI) in the mid-nineties paved the way for the introduction of (highly effective) combination antiretroviral therapy (cART), that mostly consisted of 2 NRTIs in combination with either a PI or a NNRTI[14]. cART prevented the development of resistance, strikingly improving efficacy of HIV treatment[14,16,17]. The introduction of cART dramatically improved the prognosis of HIV and the term AIDS is therefore generally considered inappropriate these days to define the health status of people living with HIV (PLHIV)[16,18].

Although cART had reduced morbidity and mortality for PLHIV, drug toxicity was a major concern[19]. Lifelong cART was associated with short-term and long-term adverse events[19,20]. Class specific adverse effects were noticed. For example, PI-use was linked to lipid and metabolic disturbances with a possible increased risk for myocardial infarction[21-23] and NRTIs were linked to (severe) mitochondrial toxicity[24-28] resulting in a wide variety of clinical syndromes, including lactic acidosis, lipodystrophy, peripheral neuropathy, myopathy, cardiomyopathy, and pancreatitis[28-32]. Instead, particular adverse effects were related to the use specific drugs, such as allergy and nevirapine[33], neurotoxicity and efavirenz[34,35] and AZT was especially known for hematological toxicity such anemia or neutropenia[12,36]. These adverse effects were reduced when older NRTIs stavudine, AZT and didanosine were replaced by newer NRTIs tenofovir (TDF) and abacavir at the turn of the millennium[25]. Still these new NRTIs have been linked to kidney dysfunction and cardiovascular disease, respectively[22,37-40]. The approval of the first integrase inhibitor (INSTI) in 2007, raltegravir, extended cART regimen options for PLHIV experiencing drug toxicity[41]. However, cART toxicity remains a concern upon this day[39,40,42-44].

Thus, to offset drug toxicity, cART initiation was initially reserved for people with substantial immunosuppression indicated by CD4+ counts below 350 cells/µL[45,46]. The hallmark SMART-trail in 2006 randomized PLHIV to continuous treatment after cART initiation or cART interruption based on CD4+ count guided therapy as such lowering cART exposure. The cART interruption group showed increased mortality and morbidity from AIDS but also from non-AIDS related events such as cardiovascular disease and cancer[47]. These events were especially linked to persistent inflammation and immune dysfunction and not fully explained by the degree of immunosuppression (CD4 count)[48]. This resulted in a paradigm switch with a focus on sustained viral suppression with immune recovery, resulting in a reduction of inflammation.

Moreover, the lowest CD4 count (CD4 nadir), was found to be linked to mortality and morbidity even when viral loads were continuously suppressed, suggesting that severe immune suppression caused ongoing perturbations[48-51]. While initiation of cART improved life expectance in 1996[16], the introduction of less toxic regimes in 2008 further enhanced prognosis[52]. However, PLHIV that started cART between 1997 and 2012 still had a six times higher risk to die compared to the general population[52].

cART for all, HIV a chronic disease

Higher mortality risks for PLHIV on cART led to the START-trial investigating whether the prognosis of PLHIV could be improved when cART was given regardless of CD4+ counts[53]. The START trial indeed showed a beneficial effect of immediate initiation of cART in PLHIV with CD4+ counts above 500cells/µL[53]. These results aligned benefits for individual patients with public health benefit, as viral suppression due to cART also reduced the risk of HIV transmission to almost zero[54]. The results of the START study published in 2015 led to the recommendation by the World Health Organization (WHO), European Aids Clinical Society (EACS) and International Antiviral Society (IAS)-USA panel to expand the use of cART to all PLHIV[55-57]. Currently, the WHO estimates 38 million people are living with HIV of which 25.4 million receive cART[58].

In 2009 Timothy Brown was reported to be cured from HIV after a stem cell transplantation with a donor carrying a homozygote mutation in the CCR5 co-receptor for HIV (CCR5Δ32 genotype), which was hailed with great excitement[59]. A second patient reported to be cured from HIV followed ten years later[60]. Apart from the excitement that HIV cure was possible

after allogenic stem cell transplantation with a very specific genetic phenotype, researchers, clinicians and patients also realised that such intervention was only feasible in a very selected group of PLHIV. Still, the possibility of cure boosted research on the HIV reservoir. These studies revealed that HIV persists in multiple cell types and tissue sites, and in both quiescent and proliferating, long-lived, latently infected cells, which complicates the total eradication of the viral reservoir[61-63]. This makes cure a distinct prospect for most.

Viral suppression and cART for all PLHIV has contributed to further improvements in the life expectancy of PLHIV. Whereas 30-years ago an HIV infection was considered a terminal illness and care mainly focused on palliative care, PLHIV now have near normal life expectancies[51]. Nonetheless, HIV cure remains a distant prospect and PLHIV still have to cope with challenges of chronic inflammation, such as increased risk for cardiovascular diseases and multi-morbidity comparable to people living with diabetes[51,64]. Apart from increased risk of cardiovascular disease (CVD), the risk for malignancies, osteoporosis and neurocognitive impairment is increased in HIV[38,48,64,65]. Factors influencing development of non-AIDS co-morbidities are complex and include cART itself, coinfections (e.g. hepatitis C), nutritional status, life style factors such as smoking or timing of cART initiation[38,48,66,67]. Underlying mechanisms are incompletely understood, but hypotheses evolve around persistent inflammation, incomplete immune restoration but also increased coagulated state with platelet dysfunction and increased d-dimer levels[65]. Although early cART initiation reduces the risk for those comorbidities, PLHIV treated during acute HIV infection still experience persistent inflammation after prolonged viral suppression[68,69].

Persistent inflammation and immune dysfunction

The human immune system can be divided in an innate and adaptive immune compartment. The innate immune system consists of monocyte, macrophages, dendritic cells and platelets. The innate immune cells react non-specifically upon external stimuli producing inflammatory mediators such as cytokines and chemokines causing (systemic) inflammation. Innate pro-inflammatory processes are also involved in atherosclerosis and cardiovascular disease[70]. Pro-inflammatory monocyte subsets are increased in PLHIV even after viral suppression is reached[71,72] and increased circulating concentrations of markers of innate immunity and monocyte activation, such as soluble CD14 (sCD14), soluble CD163 (sCD163), high-sensitive C-reactive protein (hsCRP), interleukin (IL)-6 have been linked to non-AIDS comorbidities and mortality[48,65,73-80].

The adaptive immune compartment mainly consists of T-cells and B-cells. These cells mount a specific response and harbour a classic immunological memory. Despite long-term suppressive cART, CD4 restoration is often incomplete causing long-term depletion, residual activation and senescence of CD4+ cells[81,82]. These perturbations are most prominent in lymphoid tissues. For example, resident T-helper (Th) 17 cells in the gut, which are important for gut immunity and integrity, are depleted during HIV infection[83] and are not fully restored after cART initiation[84]. CD8+ T-cell activation and dysfunction is a prominent feature of uncontrolled HIV infection, but remains present despite cART treatment[81,82]. In addition, residual B-cell dysfunction contributes to impaired vaccination responses in PLHIV[85]. Furthermore, all compartments of the immune system maintain homeostasis through a complex network of direct and indirect interactions in normal conditions. However, even after prolonged viral suppression, this complex balance is not fully restored in PLHIV[86].

Finally, blood platelets are the second most numerous blood cell and traditionally known for their role in haemostasis and cardiovascular diseases. Increasing evidence indicates that platelets are key players in inflammation and host defence[87]. Despite lacking a nucleus, platelets harbour fully functional mitochondria and a full translation machinery for protein synthesis[88]. Platelets contain many different proinflammatory molecules, such as cytokines (e.g. IL-1α, IL-1β, IL-18) and chemokines (e.g. Chemokine (C-C motif) ligand 5 (CCL5), CD40L)[87]. Upon activation, platelets can release those chemokines and cytokines, but can also release plasma membrane platelet-derived microparticles (PMPs) into the circulation. PMPs are in the submicron range and are involved in haemostasis. Both PMPs and platelets itself can directly interact with leukocytes to modulate leukocyte function[87]. Multiple studies have shown that PLHIV have more activated and reactive platelets[89-93], while others report hyporeactive platelets[94,95]. Interference of cART with platelet function could partly explain these heterogenous findings. While abacavir (a NRTI) has been linked to platelet perturbations in multiple studies[96-98], others could not confirm abacavir-associated platelet dysfunction[92,99]. Additionally, our group previously found in a cross-sectional study that individuals on a raltegravir-based regimen had reduced platelet reactivity compared to those on a non-integrase inhibitor-based regimen[92].

Factors influencing inflammation

During an uncontrolled HIV infection, the level of inflammation is mostly linked to the dynamic of the HIV infection itself and viral suppression with cART significantly reduces inflammation[80]. Yet, the underlying causes of persistent inflammation after successful cART are much more complex. Several factors have been suggested to contribute to persistent inflammation in these circumstances. First of all, ongoing exposure to microbial products has been implicated to underly persistent inflammation in PLHIV, including the HIV viral reservoir, cytomegalovirus (CMV) co-infection and increases in circulating microbial products resulting from compromised intestinal integrity[65,100-105]. The establishment of the HIV reservoir occurs within days of infection and comprises of latently infected memory CD4+ T cells and a variety of functional T helper cells with replication competent virus as well as defective proviruses[106]. The duration of HIV infection and cART treatment, as well as CD4 nadir and patient-specific traits are known to influence HIV reservoir size[61].

Even though early treatment mitigates a large part of the disturbances caused by HIV, inflammation remains. [68,69] Numerous interventions to reverse persistent inflammation and dysfunction in PLHIV have been explored, including cholesterol-lowering agents[107-109], angiotensin-converting enzyme (ACE) inhibitors[109], valganciclovir (treating asymptomatic CMV)[110] and aspirin[93]. Even though some showed a decrease in inflammation, it is questionable that these interventions will be potent or targeted enough to reverse persistent inflammation and immune dysfunction. Furthermore, biomarkers (e.g. d-dimer, sCD14, sCD163, IL-6) that are currently used for assessment of immune activation and dysfunction, provide insight in only a selection of possible pathways[65]. This is likely an oversimplification of the complex nature of HIV-associated persistent inflammation and an unbiased exploration of involved pathways is required.

Human functional genomics project

The human functional genomics project (HFGP) aims to improve our understanding of the variability of immune responses in human population[111]. Exploring how this variability relates to susceptibility to immune mediated diseases and to the response to immune based therapies. Previous studies have assessed factors that are associated with the interindividual variability in human immune responses, but frequently, only a limited number of factors with a limited number of immunological responses were investigated in healthy HIV-uninfected individuals[112,113] or PLHIV[114-118]. The HFGP integrates -omics-based data (e.g. genomics, microbiome metagenomics, metabolomics, transcriptomics) with functional immune assays, platelet function and coagulation capacity in large cohorts of healthy individuals and patients. Using biostatistical integration of these data allows the prediction of novel interactions and targets for possible treatments[111]. This approach has already proven to be successful in HIV-uninfected individuals. It provided new insights on the influence of host and environment factors[119], genetic factors, or the gut microbiota on host individual cytokine responses and immune cell populations[119-124]. A similar approach may therefore unravel the determinants of persistent inflammation of PLHIV using cART and as such provide basis for new interventions targeting non-AIDS related comorbidities.

Aim of this thesis

We established a Dutch PLHIV cohort on long-term effective cART within the HFGP. The HFGP provides a methodological framework through which specific HIV related immune or nonimmune biological processes can be assessed. In this thesis we provide the first steps into this approach exploring alterations in plasmatic coagulation, platelet function and immune responses in PLHIV. Integrating and linking different (patho)physiological pathways in PLHIV and lay ground-work for larger -omics-based projects tackling new challenges in HIV care and cure, mirroring the improvements in the HIV landscape as a whole.

Outline thesis

In **Chapter 2-6**, we used the HFGP methodology to answer specific questions related to HIV. In **Chapter 2**, we investigated the function and phenotype of peripheral blood mononuclear cells (PBMC) by using an integrative approach of functional and transcriptional analyses with a focus on the inflammatory properties of monocytes. We linked these monocyte properties to markers of persistent inflammation (e.g. IL-6, sCD14, sCD163, hsCRP). We also explored underlying mechanisms of this inflammatory monocyte profile, including the possible contribution of exposure to microbial translocation, CMV seropositivity and HIV reservoir size.

The relation between changes in the adaptive immune system and the inflammatory response is explored in **Chapter 3**. We used flow cytometry to comprehensively assess the WBC composition of PLHIV and compared the architecture of circulating immune cells with data generated in uninfected healthy controls. We explored the contribution of demographic and lifestyle related factors, as well as more specific HIV-related factors such as the HIV reservoir. Finally, we explored functional consequences of alterations in circulating adaptive immune cells by measuring the ex vivo production capacity of T-cell derived cytokines (IL-17, IL-22, and interferon (IFN)-γ).

Clonal haematopoiesis, caused by somatic mutations, emerges as a new risk factor for cardiovascular disease and haematological cancers in the general population[125,126]. The incidence of these mutations is unknown in PLHIV. In **Chapter 4**, we analysed the occurrence of somatic mutations and clonal haematopoiesis in PLHIV and studied whether changes could be associated with HIV-specific traits, HIV reservoir size and activity, inflammation and coagulation. Furthermore, we explored possible mutational signatures specifically involved in the development of somatic mutations in PLHIV.

The interaction between inflammation and coagulation is well known[127]. In **Chapter 5**, we studied whether plasmatic coagulation capacity was different between PLHIV and uninfected controls. Furthermore, we explored the effect of cART, most notably abacavir, and inflammation on these parameters.

Platelets play an important role in inflammation and development of CVD[87]. Platelets do not have a nucleus but possess mitochondrial DNA[88]. Mitochondrial toxicity is the main underlining mechanism behind NRTI toxicity[27]. In **Chapter 6**, we determined whether mitochondrial DNA levels, a measure of mitochondrial toxicity[128], of platelets differ between PLHIV and healthy controls, what the possible functional consequences are and the possible role of prior antiretroviral therapy, more specifically AZT.

In **Chapter 7-9**, I describe additional studies outside the HFGP, to further investigate mechanisms linking platelet function to (persistent) inflammation. In **Chapter 7**, we performed a randomized controlled trial to study whether a switch to a raltegravir-based regimen leads to a reduction in platelet reactivity in PLHIV. This study was performed to confirm results from a previous cross-sectional study of our group which showed that PLHIV using a raltegravir based regimen experienced lower platelet reactivity[129].

Platelets may exert their immune modulating effect through interaction with other immune cells[87]. In **Chapter 8**, we explored the effect of platelets and its microparticles on the differentiation and pro-inflammatory traits of regulatory T cells in vitro and in PLHIV.

To investigate the effect of platelets on systemic inflammation, we aimed to study the effect of platelet inhibition on inflammation using a well-validated human in vivo model of systemic inflammation (endotoxemia model)[130] in **Chapter 9**.

A summary of the findings and implications for future directions of this thesis are summarized in **Chapter 10**.

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Chapter 2

Chronic HIV infection induces transcriptional and functional reprogramming of innate immune cells

Wouter van der Heijden#, Lisa Van de Wijer#, Farid Keramati, Wim Trypsteen, Sofie Rutsaert, Rob ter Horst, Martin Jaeger, Hans Koenen, Hendrik Stunnenberg, Irma Joosten, Paul Verweij, Jan van Lunzen[6], Charles Dinarello, Leo Joosten, Linos Vandekerckhove, Mihai Netea, André van der Ven#, Quirijn de Mast#

# Equal contribution

JCI Insight 2021; 10.1172/jci.insight.145928

Abstract

Chronic inflammation and immune dysfunction play a key role in the development of non-AIDS related comorbidities. The aim of our study was to characterize the functional phenotype of immune cells in people living with HIV (PLHIV). We enrolled a cross-sectional cohort study of PLHIV on stable antiretroviral therapy and healthy controls. We assessed *ex vivo* cytokine production capacity and transcriptomics of monocytes and T-cells upon bacterial, fungal and viral stimulation. PLHIV exhibited an exacerbated pro-inflammatory profile in monocyte-derived cytokines, but not in lymphocyte-derived cytokines. Particularly, the production of the IL-1β to imiquimod, *E. coli* LPS and *Mycobacterium tuberculosis* was increased, and this production correlated with plasma concentrations of high-sensitivity C-reactive protein and soluble CD14. This increase in monocyte responsiveness remained stable over time in subsequent blood sampling after >1year. Transcriptome analyses confirmed priming of the monocyte IL-1β pathway, consistent with a monocyte trained immunity phenotype. Increased plasma concentrations of β-glucan, a well-known inducer of trained immunity, were associated with increased innate cytokine responses. Monocytes of PLHIV exhibit a sustained pro-inflammatory immune phenotype with priming of the IL-1β pathway. Training of the innate immune system in PLHIV likely plays a role in long-term HIV complications and provides a promising therapeutic target for inflammation-related comorbidities.

Introduction

Persistent inflammation and immune dysfunction play an important role in the development of non-AIDS related comorbidities in people living with HIV (PLHIV). Those include cardiovascular disease (CVD), neurocognitive dysfunction and cancer [1-4]. Reducing inflammation is considered to be an attractive therapeutic target to reduce the burden of non-AIDS related events. However, immune dysfunction and persistent inflammation in PLHIV are complex processes that are still incompletely understood. These involve changes in the adaptive immune system, including dysfunction and senescence of T-cell lymphocytes [5], as well as changes in the innate immune system. Mediators of the latter, including soluble (s)CD14, sCD163 and high-sensitive C-reactive protein (hsCRP) are indicative of persistent immune activation and are associated with non-AIDS related events [6-8].

Ongoing exposure to microbial products, for example by cytomegalovirus virus (CMV), continuing HIV replication or microbial translocation, may be one of the drivers of persistent inflammation [2,9-12]. Increased inflammation can also result from a functional adaptation of innate immune cells induced by epigenetic reprogramming; a process termed 'trained immunity' [13-16]. Trained immunity is recognized to play an important beneficial role in host defense processes, but may also contribute to conditions like atherosclerosis or type 2 diabetes mellitus [17,18]. Whether trained immunity contributes to persistent inflammation in PLHIV is currently unknown.

The Human Functional Genomics Project (HFGP) aims to investigate variation in the immune responses [19]. Studies in HFGP cohorts in healthy individuals have yielded different novel insights in the genetic and non-genetic regulation of inflammatory cytokine responses in response to microbial ligands [20-23]. We established a HFGP cohort of Dutch PLHIV and in this cohort we describe alterations in the function and phenotype of peripheral blood mononuclear cells (PBMC) by using an integrative approach of functional and transcriptional analyses. We also explored underlying mechanisms of the inflammatory monocyte profile, including the possible contribution of exposure to microbial translocation, CMV seropositivity and HIV reservoir size.

Results

Characteristics

A total of 211 PLHIV on combination antiretroviral therapy (cART) and 56 HIV-uninfected controls were included in the analyses. Baseline characteristics are shown in Table 1. Compared to the healthy controls, PLHIV were more often male (91.5% vs. 60.7%; p<0.001) and of older age (median [IQR] age of 52.5[13.2] vs 30.0 [27.1] years, p<0.0001). The median (IQR) duration of cART use in PLHIV was 6.6yr (4-12yr) and 89% were virally suppressed (HIV viral copies ≤50 copies/mL) for more than one year.

Circulating inflammatory markers and innate cytokine responses

We first measured concentrations of circulating inflammatory markers. Compared to healthy controls PLHIV had significantly higher concentrations of sCD14, hsCRP, IL-18, IL-18 binding protein (IL-18BP) and IL-6 (Figure 1A-G). These differences remained after adjusting for age, sex, body mass index (BMI), or seasonality (Figure 1A). Concentrations of adipokines were similar between groups after correction (Figure 1A).

Next, we analyzed functional changes in the innate and adaptive immune response. PBMCs were incubated with 12 stimuli (4 bacterial, 3 fungal, 1 viral and 6 Toll like receptor (TLR) ligands), followed by measurement of different monocyte-derived (IL-6, IL-1β, TNFα) and lymphocyte-derived (IL-17, IL-22, IFNγ) pro-inflammatory cytokines, as well as anti-inflammatory cytokines (IL-10, IL-1Ra) in the supernatant. We observed markedly increased monocyte-derived cytokine responses in PLHIV, especially IL-1β responses upon stimulation with LPS (TLR4), imiquimod (TLR7) and *Mycobacterium tuberculosis* (Figure 2A,C,D). Production of TNFα and IL-6 were also increased in PLHIV (Figure 2A,E), except for lower production in response to *Staphylococcus aureus* (TNF-α) and *Candida albicans* (IL-6; Figure 1F,G). This increased pro-inflammatory cytokine response in PLHIV was not associated with a concurrent increase in the anti-inflammatory cytokines IL-1Ra or IL-10. However, the release of IL-1Ra by unstimulated cells was higher in PLHIV (Figure 2H), which is consistent with increased basal IL-1β production (Figure 2A). The number of monocytes in the isolated PBMCs (Supplemental Figure 1A-B) may influence cytokine production, but adjusting for the monocyte fraction in PBMCs did not significantly alter the outcome (Supplemental Figure 1C. T-cell-derived cytokine responses were generally lower for most stimuli in the PLHIV cohort (Supplemental Figure 2), but these differences were abrogated following adjustment for age, sex and seasonality (Figure 1A), except for IFNγ responses to *M. tuberculosis* and C. albicans hyphae (Figure 2A-B). There were no associations between cytokine production capacity and smoking or different HIV-specific traits, including recent CD4 cell count, CD4/8 cell ratio, CD4 nadir, cART-regimen or co-medication (metformin, statins or aspirin) (Supplemental Figure 3).

Table 1. Baseline characteristics

	PLHIV (n=211)	Healthy controls (n=56)
Sex (% Male)	193 (91.5)	34 (60.8)[A]
Age (years, mean (SD))	51.4 (10.8)	39.9 (17.3)[B]
BMI (kg/m², mean (SD))	24.5 (3.6)	24.1 (3.1)
HIV infection duration (years, median [IQR])	8.5 [4.9, 14.2]	
Way of transmission (%)		
Heterosexual	9 (4.3)	
IDU	3 (1.4)	
MSM	159 (75.4)	
Other/unknown	40 (19.0)	
CD4 nadir (10^6 cells/mL; median [IQR])	250.0 [135.0, 360.0]	
CD4 count (10^6 cells/mL; median [IQR])	660.0 [480.0, 810.0]	
HIV load below 200 copies/mL (n (%))	211 (100)	
HIV load below 50 copies/mL for >1yr (n (%))	188 (89%)	
CD4/CD8 ratio (median [IQR])	0.8 [0.6, 1.1]	
cART duration (years; median [IQR])	6.6 [4.2, 11.8]	
cART regimen		
NRTI-use (%)	203 (96.2)	
NtRTI-use (%)	99 (46.9)	
NNRTI-use (%)	63 (29.9)	
PI-use (%)	32 (15.2)	
Maraviroc-use (%)	3 (1.4)	
INSTI-use (%)	141 (66.8)	
Active smoking, n/N (%)	63 (29.9)	2 (3.6)[B]
Heavy drinking (%)*	28 (13.3)	11 (19.6)
Regular substance use, (%)**	61 (28.9)	3 (5.4)[B]
Hypercholesterolemia (%)	58 (27.5)	
Hypertension (%)	41 (19.4)	
Diabetes Mellitus (%)	9 (4.3)	
No cardiovascular risk factors (%)	50 (23.7)	
Statins (%)	58 (27.5)	
Aspirin (%)	19 (9.0)	
Metformin (%)	9 (4.3)	

BMI: body mass index. IDU: intravenous drug-use. MSM: men who have sex with men. cART: combination antiretroviral therapy. NRTI: Nucleoside reverse transcriptase inhibitor. NtRTI: nucleotide reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor. INSTI: integrase inhibitor. *Classified according to the CDC definition: for men, ≥15 drinks per week and for women, ≥8 drinks/week. http://www.cdc.gov/alcohol/faqs.htm#heavyDrinking (page accessed April 22, 2020, Page last reviewed: January 15, 2020, Content source: Division of Population Health, National Center for Chronic Disease Prevention and Health Promotion, Centers for Disease Control and Prevention). **Defined as use of any psychoactive substance (with the exception of alcohol and tobacco) during periods ≥ 1 time per week including ≥ 1 time during the 30 days prior to the study visit. [A] Significantly different between cohorts using Students T-test. [B] Significantly different between cohorts using Chi-square test.

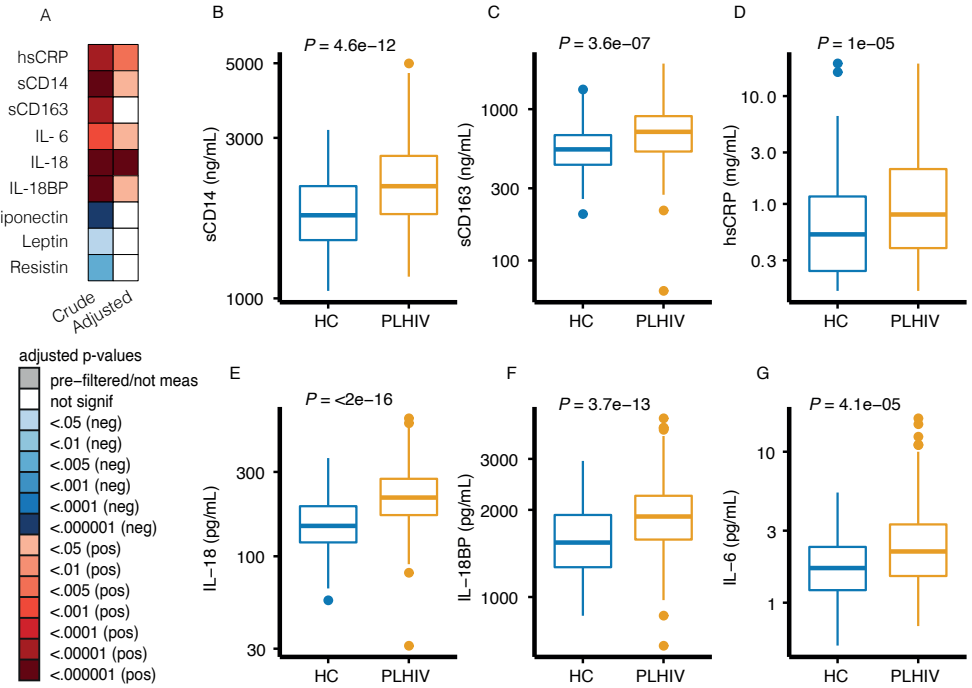


Figure 1. Circulating factors in people living with HIV (PLHIV) vs uninfected healthy controls (HC). (**A**) Circulating factors in PLHIV and uninfected controls. Crude model is linear regression after inverse rank-based transformation. Adjusted model included age, sex, and seasonality as covariates. Red depicts the marker is significantly increased in PLHIV; blue depicts the marker is decreased in PLHIV compared to healthy controls. All P values are FDR corrected (Benjamini-Hochberg method). (**B-G**) Boxplots depicting circulating factors of inflammation stratified by cohort, PLHIV (blue), uninfected controls (yellow). P values are calculated using Student's t test. Boxplots are depicted according to Tukey with median (line), interquartile range (edge of boxplot), range (whiskers) and outliers 3 times interquartile range are depicted as dots. All plots include data from PLHIV (n = 211) and HC (n = 56). hsCRP: high-sensitivity C-reactive protein; sC14: soluble CD14; sCD163: soluble CD163; IL-6: interleukin-6; IL-18: interleukin-18; IL-18BP: interleukin 18 binding protein.

Next, we assessed associations between innate cytokine responses, circulating inflammatory markers, and monocyte phenotypes (Figure 2A-I). Increased *ex vivo* cytokine responses were associated with persistent inflammation. For example, we found positive associations between IL-6 responses and circulating hsCRP (Figure 2A,I), and between LPS induced IL-1β responses and plasma sCD14 and IL-18BP (Figure 2A,D). In addition, non-classical and intermediate monocyte subsets (Figure S4A-F), which are considered pro-inflammatory monocyte phenotypes, correlated with higher IL-1β and IL-6 production after LPS or imiquimod, respectively (intermediate monocytes vs LPS-IL-1β R=0.23, p<0.001; Figure 2A-C) [24]. Taken together, we show that PLHIV have an altered innate immune profile with markedly elevated monocyte cytokine responses, particularly IL-1β, which correlates with blood biomarkers of persistent inflammation.

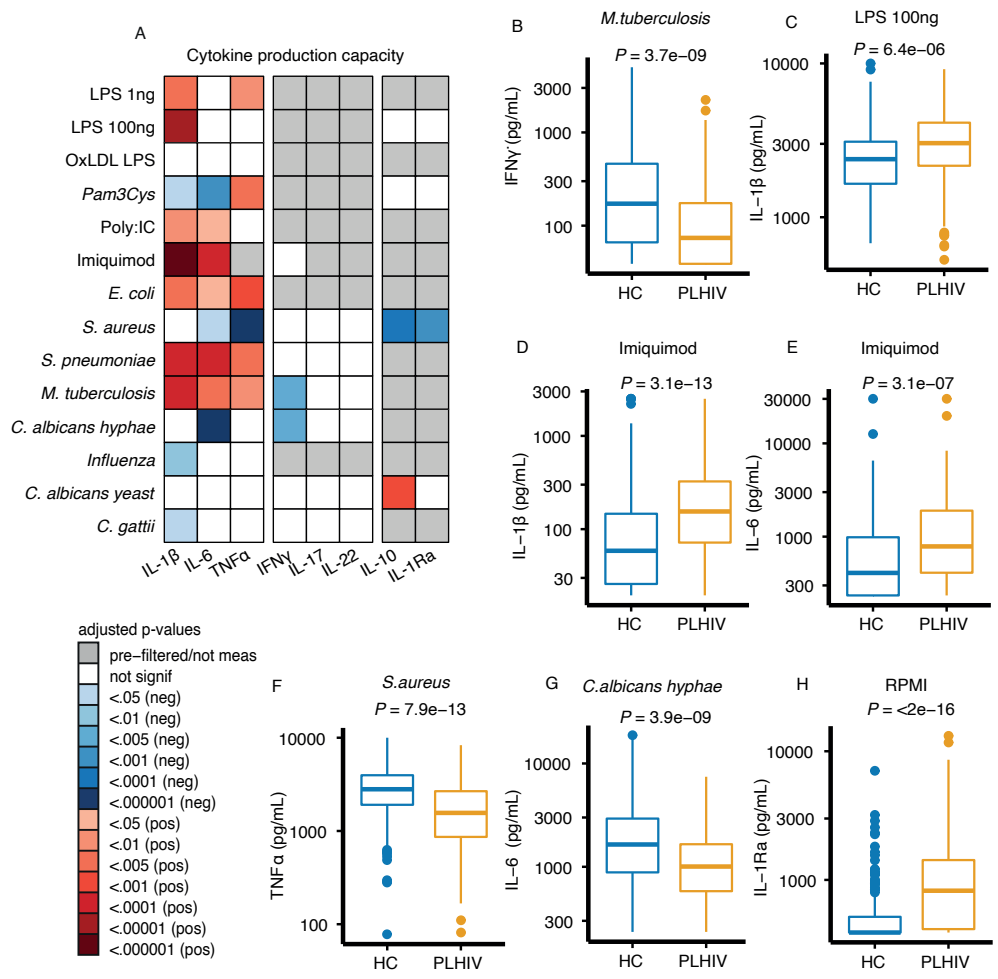


Figure 2. Cytokine production capacity in PLHIV vs uninfected healthy controls (HC). (**A**) *Ex vivo* cytokine production capacity between PLHIV and HC after 24hr (in case of IL-1β, TNFα, IL-6, IL-10 and IL-1Ra) and 7 days stimulation (IL-22, IL-17 and IFNγ). FDR-corrected (Benjamini-Hochberg method) P values are depicted from an adjusted model included age, sex, seasonality as covariates. Red depicts significantly higher in PLHIV, blue depicts lower in PLHIV compared to HC. (**B-H**) boxplots depicting *ex vivo* cytokine production capacity stratified by cohort. PLHIV depicted in blue and uninfected controls in yellow. P values, depicted in boxplots were calculated using Student's t test after log-transformation. All boxplots are depicted according to Tukey with median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. All plots include data from PLHIV (n = 211) and HC (n = 56). LPS: lipopolysaccharide; oxLDL: oxidized low-density lipoprotein; Pam3Cys: synthetic Toll-Like-Receptor (TLR)2 ligand; Poly IC: TLR3 ligand; imiquimod (IMQ): TLR7 ligand. RPMI: Roswell Park Memorial Institute medium.

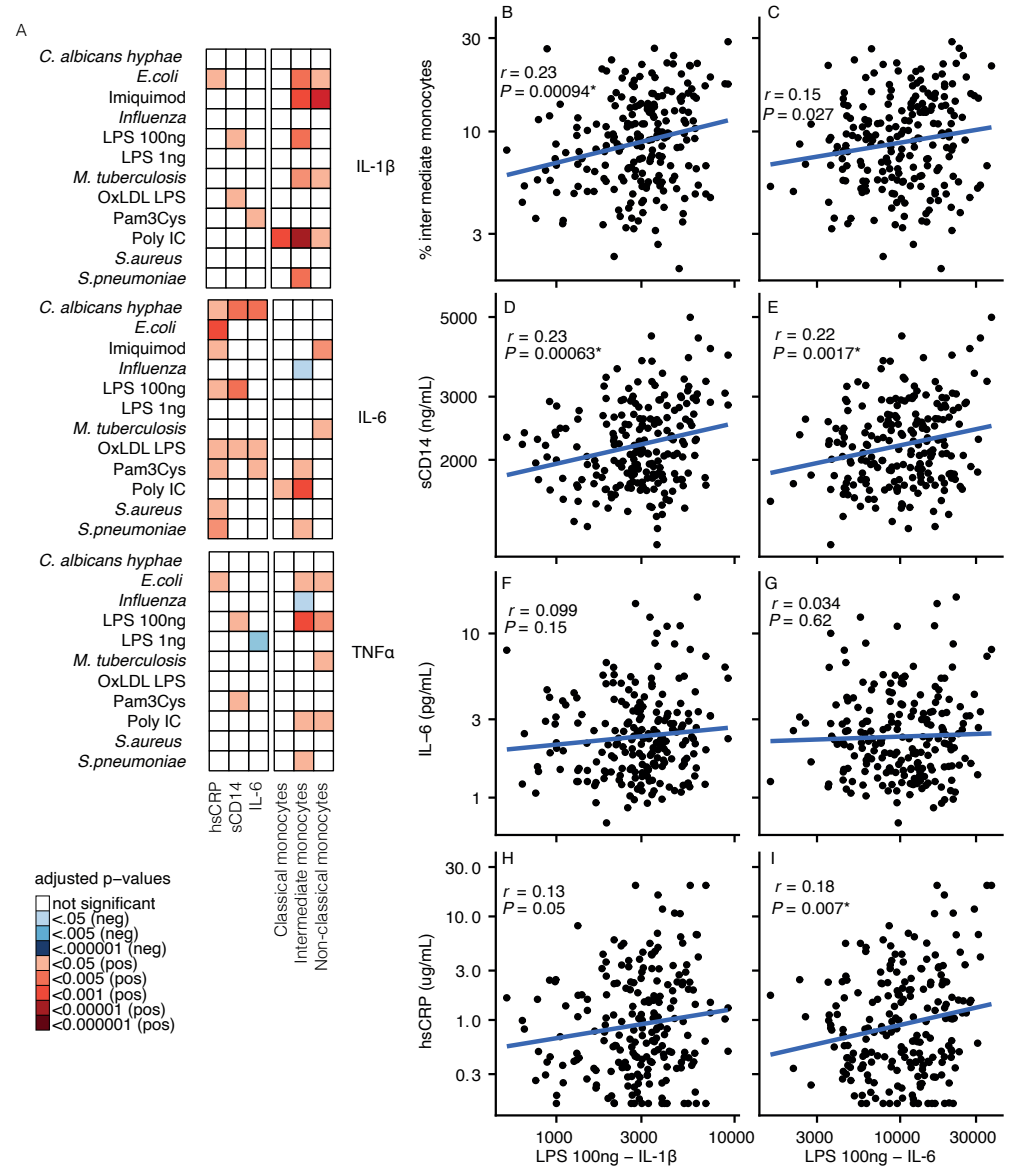


Figure 3. Cytokine production capacity vs circulating factors. (**A**) Correlation plot with adjusted p-value shown after correcting for age, sex, seasonality as covariates. Red depicts a significant positive correlation and blue depicts a negative correlation within people living with HIV (PLHIV). All P values were FDR corrected per circulating factor. (**B-I**) Correlation plot without cofactor adjustment. Pearson's coefficient (r) with P value after log-transformation are shown. All plots include data from PLHIV (n = 211) and HC (n = 56). LPS: lipopolysaccharide; oxLDL: oxidized lipodensityprotein; Pam3Cys: synthetic Toll-Like-Receptor (TLR)2 ligand; Poly IC: TLR3 ligand; imiquimod (IMQ): TLR7 ligand. hsCRP: high-sensitivity C-reactive protein; sC14: soluble CD14; sCD163: soluble CD163; IL-6: interleukin-6; IL-18: interleukin-18; IL-18BP: interleukin 18 binding protein. *P < 0.05 after FDR-correction.

Increased IL-1β production capacity of PLHIV is stable over time

We then assessed the longevity of the elevated monocyte-derived cytokine responses. We resampled 28 male PLHIV, aged >45yr after >1yr and stratified them on the basis of their initial IL-1β production capacity in a group of low (lowest quartile) and high (highest quartile) IL-1β producers. Fourteen age and sex-matched uninfected controls were enrolled concurrently (Supplemental Table 1). Upon resampling, those assigned to the high IL-1β producer group still exhibited higher IL-1β and IL-6 production compared to participants in the low IL-1β producer group and matched healthy controls, suggesting that the enhanced monocyte responsiveness is stable over time (Figure 4A-B). TNF-α followed a similar, but non-significant pattern (*P* = 0.075; Figure 4C). To examine if the presence of T-lymphocytes affected the outcome, we isolated monocytes using magnetic beads (purity >95%, Supplemental Figure 4G) and repeated the stimulation experiments. We found that IL-1β production remained increased, suggesting that the elevated monocyte-derived cytokine response is due to an enhanced functional state of the monocytes themselves.

Enhanced pro-inflammatory functional state of monocytes

Based on the previous findings, we postulated that a trained immunity functional phenotype contributes to the enhanced monocyte responsiveness in PLHIV. Hence, we isolated monocytes from eight PLHIV and four age and sex matched uninfected controls and analyzed their transcriptomes by RNA sequencing. Using principal component analysis (PCA), we observed clustering of the PLHIV and controls (Figure 5A). This clustering was even more pronounced when monocytes were differentiated towards macrophage phenotype by culture for 24hr in serum-free medium (RPMI; Figure 5B) and was also observed using hierarchical clustering (Figure 5C). Gene ontology (GO) analysis of 303 upregulated and 30 downregulated genes (Supplemental data) showed that pro-inflammatory pathways were upregulated in PLHIV, including the 'inflammatory response', 'regulation of innate immune response' and 'IL-1β production' pathways (Figure 5D-E). Among the upregulated genes in these pathways were intracellular signaling proteins and inflammasome related molecules (e.g. *NLRP3*, *STAT1*), cytokines (e.g. *IL1B*, *CCL2*, *MMP9*, *IL1RN*), and pattern-recognition receptors (e.g. *TLR2*, *TLR4*, *TLR7* and *NOD2*), underlining the broad upregulation of inflammatory pathways in PLHIV on long-term cART.

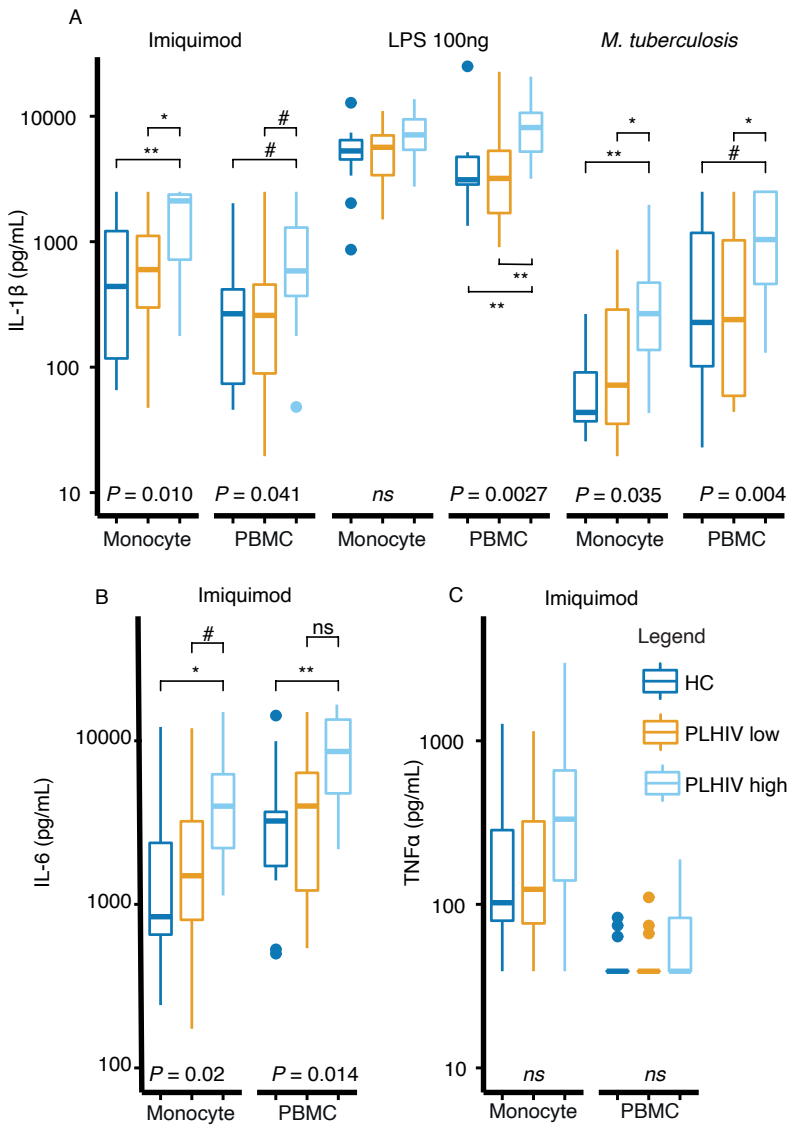


Figure 4. *Ex vivo* cytokine production capacity of monocytes in a validation cohort. (**A**) IL-1β production upon 24h stimulation with imiquimod (1ug/mL), lipopolysaccharide (LPS; 100ng/mL) or *M. tuberculosis* (1ug/mL) in PBMCs or monocyte only culture (magnetic beads CD14+ isolation). (**B**) IL-6 production upon imiquimod stimulation. (**C**) TNFα production upon imiquimod stimulation. All data are stratified by healthy controls (HC; n = 14), people living with HIV (PLHIV) low initial IL-1β producers (n = 13) and PLHIV high producers (n = 15). P- values in panel A-C were calculated by Student t test. All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots.

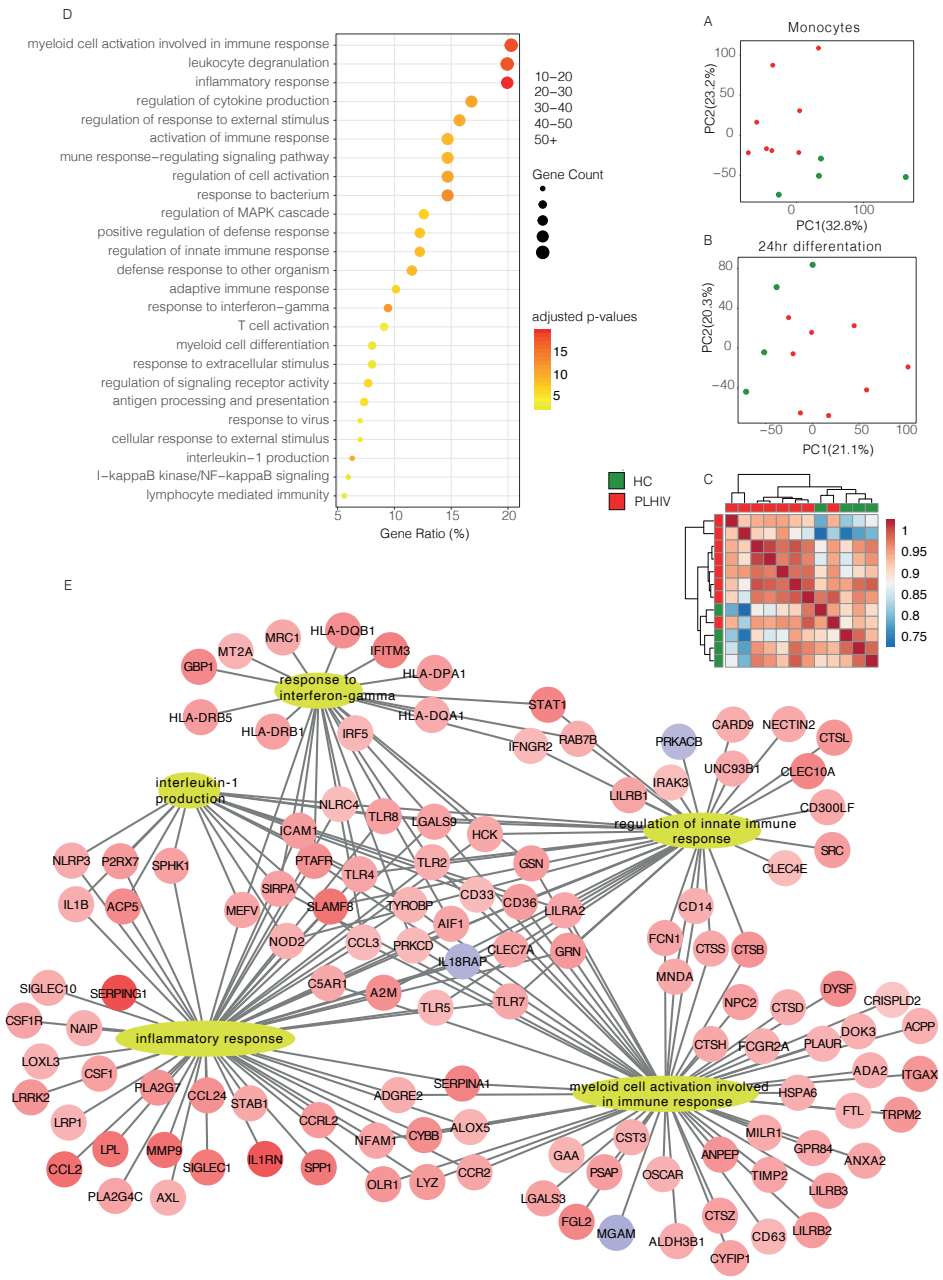


Figure 5. Transcriptome analysis of monocytes. (**A**) Principal component (PC) analysis (PCA) plot of transcriptome of monocytes from people living with HIV (PLHIV) and healthy controls (HC) directly after isolation. (B) PCA plot of transcriptome of monocytes from PLHIV and HC after 24 hours macrophage differentiation in medium only. (**C**) Hierarchical clustering plot PLHIV vs HC. (**D**) Gene ontology of differentially expressed genes (PLHIV vs HC) including adjusted P value and gene count. (**E**) Top pathways of gene ontology plot after macrophage differentiation (GO interaction terms). PLHIV: n = 8, HC: n = 4.

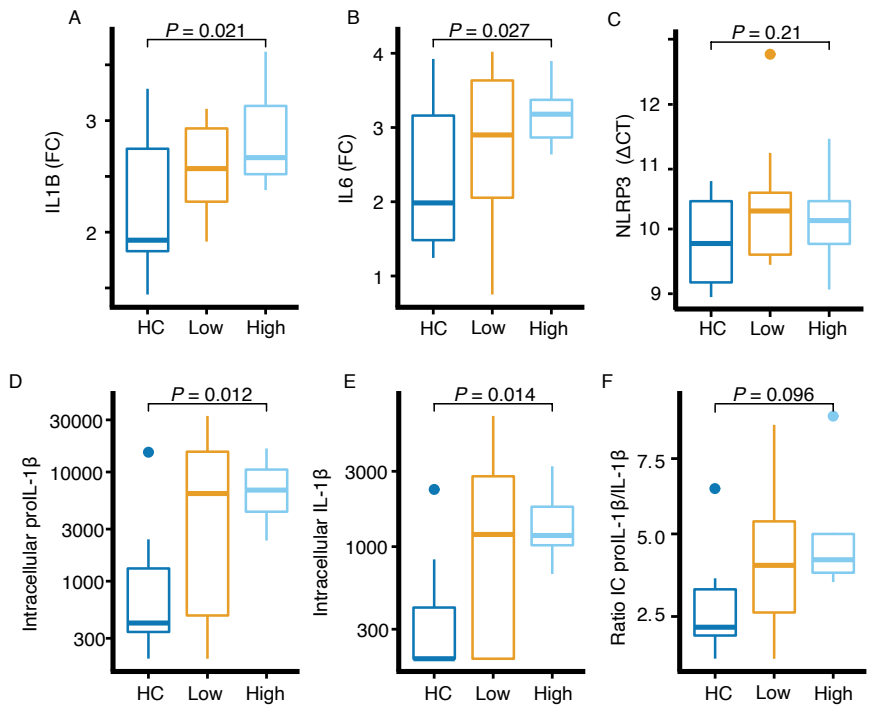


Figure 6. IL1B gene expression and intracellular (pro)IL-1β. (**A**) IL1B gene expression after imiquimod 1ug/mL stimulation depicted as fold change (FC) from medium (RPMI). (**B**) IL6 gene expression after imiquimod stimulation depicted as fold change from medium. (**C**) NLRP3 gene expression (by ΔCT) in RPMI. (**D**) Intracellular levels of proIL-1β protein after Imiquimod stimulation. (**E**) Intracellular mature IL-1β protein after imiquimod stimulation. (**F**) Ratio of intracellular proIL-1β vs IL-1β after imiquimod stimulation protein. All data are stratified by healthy controls (HC; n = 14), people living with HIV (PLHIV) low initial IL-1β producers (n = 13) and PLHIV high producers (n = 15). All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. P-values were calculated by ANOVA and subsequently by pair-wised Student's t test.

Enhanced IL-1β gene expression in monocytes of PLHIV

The release of active IL-1β from blood monocytes involves a priming signal that is mostly transcriptionally driven, which results in the synthesis of the IL-1β precursor (proIL-1β). This precursor is subsequently processed via caspase-1 into active IL-1β [25]. Using qPCR on monocytes stimulated with IMQ, we found that the *IL1B* (gene encoding for proIL-1β) RNA expression was higher in monocytes from PLHIV compared to controls (Figure 3F, *P* = 0.021). *IL6* mRNA followed a similar trend (Figure 6A, *P* = 0.027), whereas *TNFA* expression did not (data not shown, *P* = 0.74). Additionally, we measured intracellular proIL-1β and active IL-1β protein by ELISA and both were significantly elevated in PLHIV (Figure 6D-E). The inflammasome promotes the proteolytic cleavage of proIL-1β into active IL-1β, resulting in reduced proIL-1β/active IL-1β ratios in situations with increased caspase-1 activity [25]. Instead, we observed a trend towards an increased ratio of intracellular proIL-1β and active IL-1β (Figure 6F) in the high IL-1β producing PLHIV, and no differences in levels of NLRP3 expression (Figure 6C). Together, these results demonstrate that the enhanced IL-1β transcription, rather than increased processing through caspase-1, is the biological process that primarily drives the increased IL-1β production in PLHIV.

β-glucan exposure induces a pro-inflammatory monocyte phenotype

We next examined possible pathways underlying the pro-inflammatory monocyte phenotype in PLHIV. Persistent exposure to microbial ligands derived from gut microbes, CMV or HIV itself has been suggested to play a role in the persistent inflammation in PLHIV[2,9-12]. We postulated that these factors also play a role in the pro-inflammatory monocyte phenotype. We, therefore, first assessed whether innate cytokine responses were associated with parameters of the HIV viral reservoir. The HIV reservoir was assessed by analyzing CD4+ cell-associated HIV-1 DNA (CA-DNA) and CA-RNA. In virally suppressed patients, the CA-DNA roughly equals the integrated HIV-1 DNA, being replication competent or not[26], while CA-RNA is associated with recent HIV-1 transcriptional activity and serves as a proxy for the active proviral reservoir[27]. While cell associated HIV-1 CA-DNA levels in CD4+ T-cells were associated with plasma IL-6 concentrations (Figure 7A), there was no association between IL-1β cytokine responses (Supplemental Figure 5) or plasma sCD14 concentrations and HIV-1 CA-DNA or CA-RNA levels in our cohort (Figure 7B-H).

Next, we assessed associations between cytokine responses and CMV seropositivity. In total 198/211 (93.8%) of participants were CMV seropositive. There were no differences in IL-1β cytokine responses,sCD14 concentrations or HIV-1 CA-DNA between CMV seropositive vs. seronegative individuals (Figure 7I-L, Supplemental Figure 5). Another source of chronic microbial ligand exposure in PLHIV is increased microbial translocation [9]. Plasma concentrations of Intestinal fatty acid binding protein (IFABP), a marker for microbial translocation, were significantly increased in PLHIV compared to controls (Figure 7M). We found a modest negative correlation of IFABP concentrations with IL-1β cytokine responses (Figure 7N-O), but no correlation with sCD14 concentrations (Figure 7P).

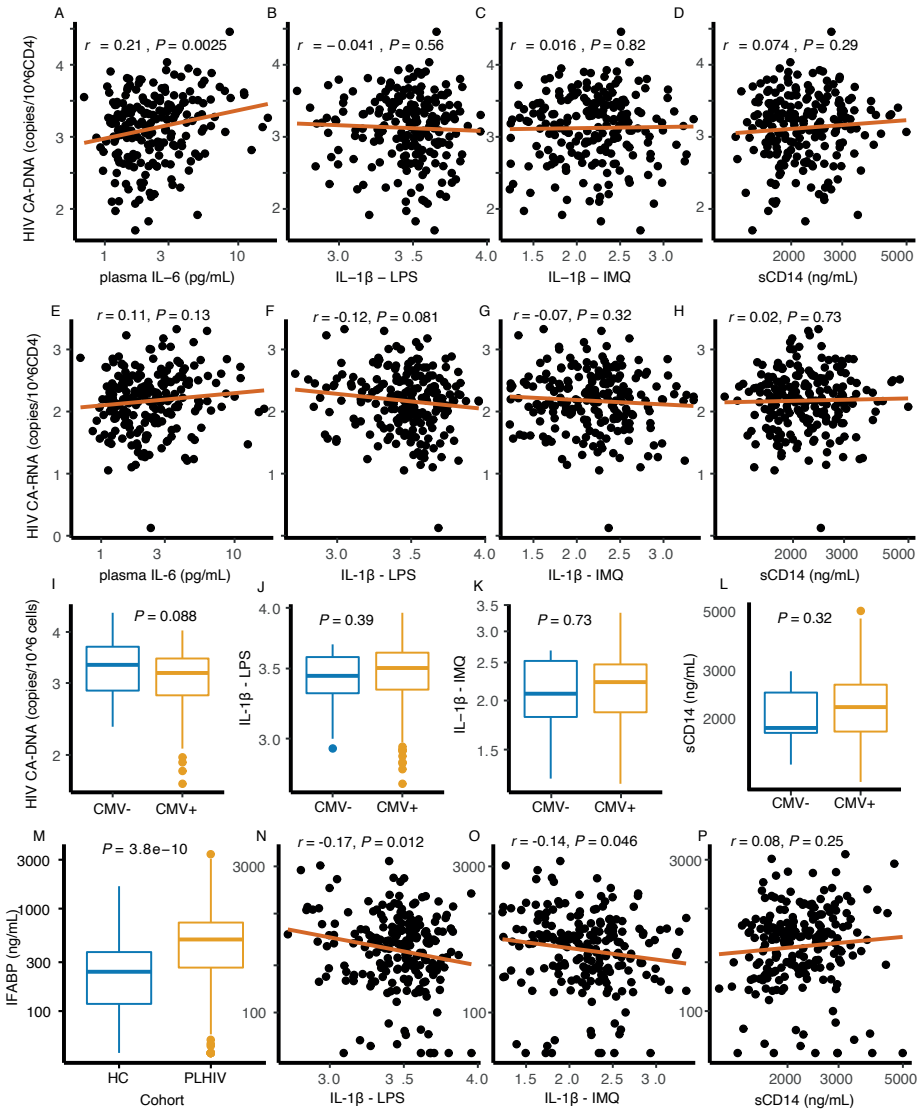
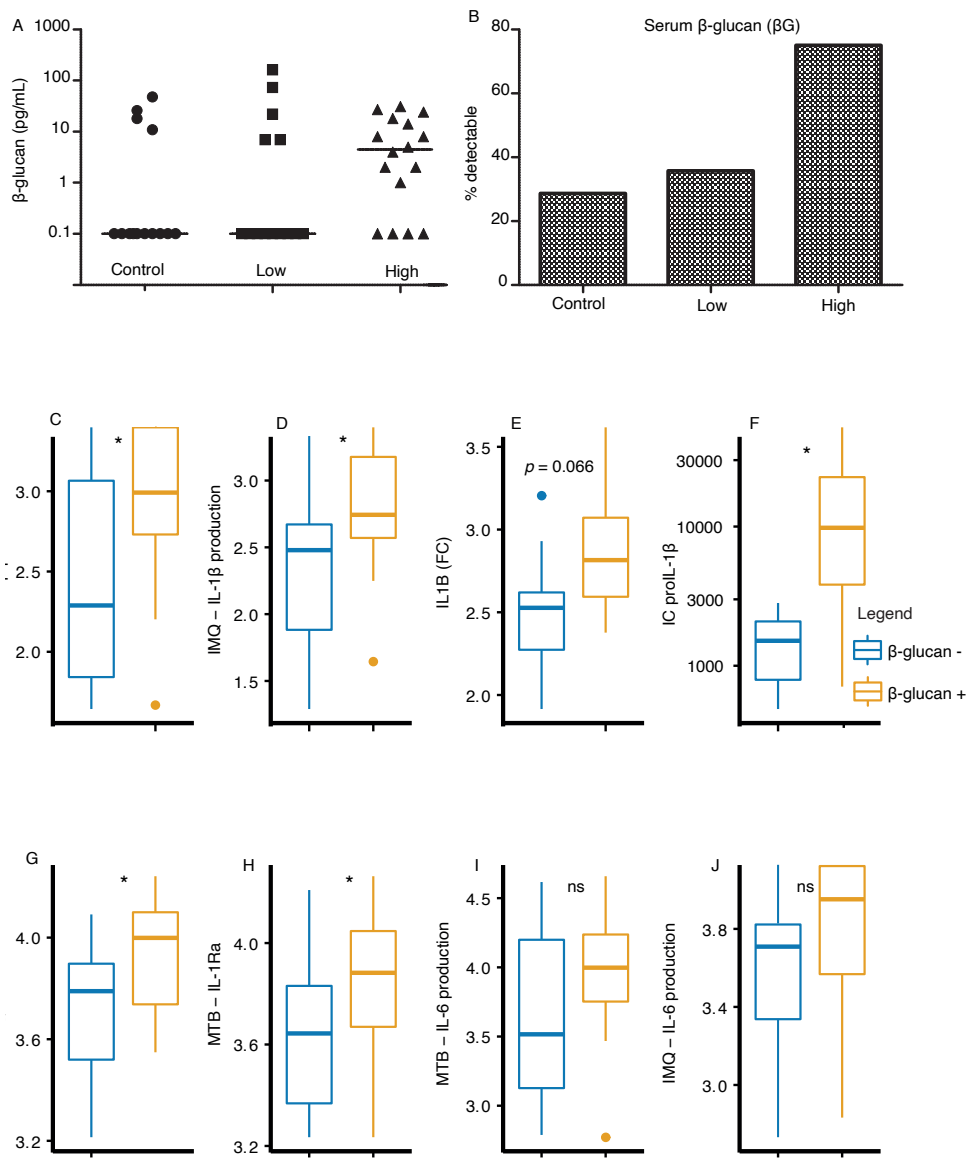


Figure 7. Parameters of HIV reservoir, CMV seropositivity and microbial integrity. (**A-D**) Log10-transformed cell associated HIV-1 DNA (CA-DNA) in CD4+ cells correlation with: (**A**) circulating IL-6 levels, (**B**) IL-1β production after LPS (100ng/mL) stimulation, (**C**) IL-1β production after imiquimod (IMQ; 1ug/mL) stimulation and (**D**) soluble CD14 plasma concentration. (**E-H**) Log10-transformed cell associated HIV-1 RNA (CA-RNA) in CD4+ cells correlation with: (**E**) circulating IL-6 levels, (**F**) IL-1β production after LPS stimulation, (**G**) IL-1β production after imiquimod stimulation and (**H**) soluble CD14 plasma concentration. (**I-L**) Stratified by Cytomegalovirus (CMV) seropositivity: (**I**) Log10-transformed cell associated HIV DNA (CA-DNA) in CD4+ cells, (**J**) IL-1β production after LPS stimulation, (**K**) IL-1β production after imiquimod stimulation and (**L**) soluble CD14 plasma concentration. (**M**) Intestinal fatty-acid binding protein (iFABP) in plasma, a marker of intestinal integrity, between healthy controls (HC; n = 56) and people living with HIV (PLHIV; n = 211). (**N-P**) Plasma iFABP concentration correlation with: (**N**) IL-1β production after LPS stimulation, (**O**) IL-1β production after imiquimod and (**P**) soluble CD14 plasma concentration. All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. Pearson's coefficient (r) after log-transformation is shown in correlation plots. Data in boxplots are analyzed using Student's t test after log10-transformation. All plots depict data from PLHIV only (n = 211) unless otherwise stated.

Increased intestinal translocation may also increase circulating concentrations of β-glucan, which is a well-known inducer of trained immunity [28]. We therefore determined serum β-glucan concentrations in the subsequent cohort of 28 PLHIV and 12 controls. Compared to controls (4/14; 29%), a significantly higher proportion of PLHIV (16/28; 57%), and especially those in the high IL-1β producer group (11/15; 73%), had detectable β-glucan in serum (Figure 8A-B). Individuals with detectable serum β-glucan had elevated IL-1β production (Figure 8C-D), showed increased IL1B gene expression (Figure 8E) and increased intracellular proIL-1β upon stimulation (Figure 8F). Concurrently, IL1ra production was increased in PLHIV with detectable β-glucan concentrations (Figure 8G). A similar, non-significant trend was observed for IL-6 and TNF responses (Figure 8H-I). A similar, non-significant trend was observed for IL-6 and TNF-α responses (Figure 8I-L). Next, using a well-established in-vitro training protocol of trained immunity [29], we confirmed that pre-stimulation of adherent monocytes with β-glucan resulted in a pronounced enhancement of IL-6 release upon re-stimulation with LPS at day 6 (Figure 8M). We also investigated the effects of pre-stimulation with LPS. As previously reported, pre-stimulation with LPS induced immune tolerance (Figure 8D) [15]. Taken together, these findings suggest that circulating β-glucan is a possible driver of the pro-inflammatory immune phenotype of monocytes in PLHIV.



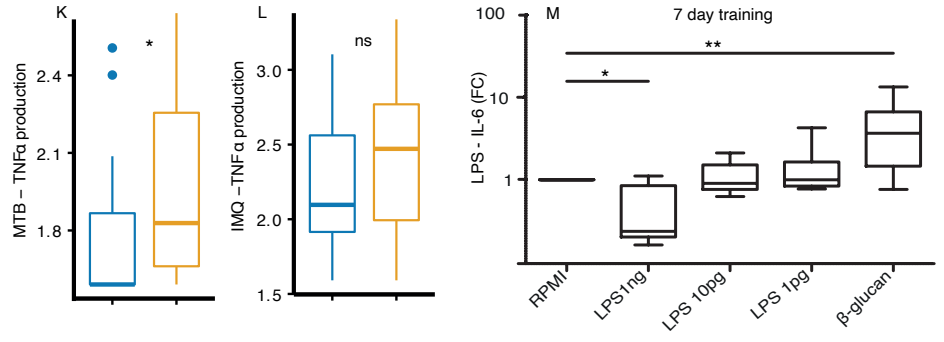


Figure 8. β-glucan induces a pro-inflammatory phenotype in monocytes. (**A**) β-glucan (βG) in serum stratified by healthy controls (control), people living with HIV (PLHIV) with low or high initial IL-1β response. (**B**) percentage of detectable levels of β-glucan in serum, stratified by control, PLHIV with high and low initial IL-1β response. (**C-D**) IL-1β production after 24h stimulation with imiquimod (**C**; 1ug/mL) or Mtb (**D**; 1ug/mL) in PLHIV stratified by detectable β-glucan levels. (**E**) IL1B gene expression after imiquimod stimulation depicted as fold change (FC) from medium (RPMI). (**F**) Intracellular levels of proIL-1β protein after Imiquimod stimulation. (**G-H**) IL1Ra production after 24h stimulation with imiquimod (**G**) or Mtb (**H**) in PLHIV stratified by detectable β-glucan levels. (**I-J**) IL-6 production after 24h stimulation with imiquimod (**I**) or Mtb (**J**) in PLHIV stratified by detectable β-glucan levels. (**K-L**) TNFα production after 24h stimulation with imiquimod (**K**) or Mtb (**L**) in PLHIV stratified by detectable β-glucan levels. (**A-L**) PLHIV n = 28, healthy controls (HC) n = 14. All boxplots are depicted according to Tukey; median (line), interquartile range (edge of boxplot), range (whiskers) and outliers three times interquartile range are depicted as dots. Data were analyzed using Student's t test after log10-transformation. (**M**) Initial training with either LPS (TLR4 ligand), β-glucan (10ug/mL) or medium only (RPMI + 10% serum) was performed for 24h at day 1. Thereafter a 5-day resting period in medium only (supplemented by 10% serum), at day 6 adherent monocytes were restimulated with LPS 10ng/mL. IL-6 was measured in the supernatant and fold change from training with medium only are depicted. Data shown are from three separate experiments (n=9). Data were analyzed using Wilcoxon matched pairs signed-rank test. * $P < 0.05$ ** $P < 0.01$.

Discussion

In this study, we investigated the functional phenotype of circulating immune cells in a cohort of PLHIV on stable cART. We show that PLHIV exhibit a sustained elevation in monocyte cytokine responsiveness, whereas lymphocyte-derived cytokine responses were mostly unaffected. Our immune profiling data, which included transcriptome analysis of circulating monocytes, further showed an increased expression and activation of the IL-1β pathway. HIV infection is associated with increased microbial translocation [9] and we identified a, so far unrecognized, role for elevated circulating β-glucan concentrations in the altered inflammatory monocyte phenotype in PLHIV.

These data corroborate findings from earlier, smaller studies in PLHIV, which reported an increased production of pro-inflammatory cytokines to stimulation with ligands of TLR4 [30,31] or TLR7 [32,33], or *M. tuberculosis* [34]. The increased monocyte-derived cytokine responsiveness in our PLHIV cohort was associated with increased plasma concentrations of different circulating inflammatory markers, such as hsCRP, sCD14, IL-18 and IL-6, suggesting a functional link between changes in the innate immune profile and systemic inflammation in PLHIV. Remarkably, HIV infection had little effect on lymphocyte-derived cytokine responses, despite the fact that alterations in T-cell immune phenotype have been described in long-term viral suppressed PLHIV[35,36]. Previous research in HFGP cohorts of HIV-uninfected individuals showed that age, environmental, microbial and genetic factors have a clear impact on the function of circulating T-cells [21-23,37].

The observed intrinsic pro-inflammatory phenotype of monocytes in PLHIV was stable over time and associated with upregulation of pro-inflammatory pathways, most notably the IL-1β pathway. This corroborates a recent study showing a pronounced pro-inflammatory phenotype, including upregulation of *IL1B*, of monocyte derived macrophages in PLHIV [38]. Long-term epigenetic reprogramming of monocytes in response to microbes has been termed 'trained immunity'. This functional adaptation of monocytes enables a greater response when subjected with a secondary stimulus as protective mechanism against a secondary infection [14-16]. Nonetheless, a trained immunity phenotype of monocytes has also been related to conditions such as cardiovascular diseases and diabetes [39-42]. In PLHIV, altered epigenetic profiles such as DNA methylation have been reported [43,44{Zhang, 2016 #4465,45]. Specific DNA methylation patterns in PLHIV were associated with progressive aging and non-AIDS related co-morbidities, such as insulin resistance, neurocognitive disorders and chronic kidney disease [17,46-48]. Interestingly, HIV/Simian immunodeficiency virus (SIV) DNA vaccination was recently shown to induce a trained immunity phenotype *in vivo* through upregulation of IL-1β related genes [49]. This upregulation correlated with protection against subsequent SIV infection in macaques. Furthermore, HIV-1 itself has also been shown to increase IL-6 production via epigenetic modification of STAT3 in microglial cells [50]. Although epigenetic modification has been shown to underlie the trained immunity phenotype, we did not determine epigenetic changes in the current study. Future studies are warranted to investigate the epigenetic processes underlying the trained immunity phenotype reported here.

Another question concerned the identification of the possible mechanisms responsible for the observed changes in innate immune responses. CMV [10], microbial translocation [9], and HIV reservoir [11] have each been related to persistent inflammation in PLHIV. However, our data did not show a positive correlation between monocyte responsiveness and CMV seropositivity, levels of IFABP (microbial translocation marker), or parameters of the HIV reservoir. Although, we do not exclude a possible accessory role for these markers of microbial exposure, we did find an association of circulating β-glucan concentrations and IL-1β responsiveness. β-glucan is a component of the cell wall of fungi and is known to be a strong inducer of trained immunity [16]. Furthermore, IL-1β signaling is reported to be important in β-glucan induced trained immunity *in vivo* [51]. Our present data corroborate earlier reports that increased circulating β-glucan concentrations can be found in virally-suppressed PLHIV [28,52,53]. Acute HIV infection leads to an early and pronounced loss of mucosal Th17 $CD4^+$ T cells [54]. This cellular compartment is critical for regulation of mucosal host defense against *Candida* and regulation of epithelial cell permeability [55]. Increased levels of other microbial products, including LPS, have also been reported in PLHIV [28,56]. However, in contrast to β-glucan, LPS generally induces immune tolerance, even at low concentrations [15,16], a notion we confirmed *in vitro*. This notion is supported by our observation that microbial integrity, as measured by iFABP, is associated with decreased monocyte responsiveness. An HIV infection is associated with alterations in the bacterial gut microbiome composition and these changes play a role in the incidence of co-morbidities and inflammation [2,57,58]. While studies on the mycobiome in PLHIV are limited, fungal dysbiosis with a high prevalence of *Candida* species has been found in stool samples of PLHIV [59]. Furthermore, our data showed lower IL-6 and higher IL-10 production by monocytes upon *Candida* stimulation in PLHIV compared to uninfected controls, suggesting an altered fungal immune response. This defective immunity could contribute to fungal dysbiosis in virally suppressed PLHIV. Hence, our present data support the need for future studies exploring the role of the mycobiome in persistent inflammation in PLHIV, as well as possible strategies to reduce β-glucan exposure.

Reducing inflammation is considered an attractive therapeutic target to reduce the burden of cardiovascular diseases. The CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial that was carried out in HIV uninfected subjects who were at risk for a cardiovascular event. The trial revealed that specific neutralization of IL-1β reduced in the incidence of subsequent cardiovascular events and death from lung cancer [60,61]. Similarly, the anti-inflammatory drug colchicine reduced cardiovascular events in patients with coronary disease [62]. In contrast, the immunosuppressive drug methotrexate did not reduce CVD, underlining the importance of IL-1β in CVD [63]. Recent findings that canakinumab reduced atherosclerotic inflammation in PLHIV at high-risk for CVD are therefore promising [64] and support the importance of IL-1β pathway in HIV. Orally administrated drugs that inhibit the IL-1β pathway, such as inflammasome inhibitors, may form an attractive alternative [65]. This also applies to epigenetic modifying drugs, such as histone deacetylase (HDAC) inhibitors. These drugs have been shown to reduce CRP concentrations, IL-1β expression in PBMCs [66] and IL-1β production to LPS stimulation in whole blood from PLHIV [67]. Taken together, our present data support the use of interventions targeting the IL-1β pathway or reduce B-glucan exposure as an adjunctive therapy to reduce non-AIDS related co-morbidities.

A particular strength of our study is that in-depth functional analysis of adaptive and innate immune cell function in a relatively large cohort of PLHIV. The same HFGP approach was successfully used to investigate genetic, environmental and microbial factors influencing the immune system in HIV-uninfected individuals [21-23,37]. Limitations of our study include that PLHIV and control cohort differed in age and sex distribution. All analyses were therefore adjusted for these differences using multivariate analyses with sufficient sample size. Moreover, the primary findings were confirmed when we resampled a selection of age and sex matched PLHIV and controls. However, we could not correct for men who have sex with men (MSM), as this information was not available for the control group[68]. Second, the nature of our cross-sectional study does not allow to draw strong causal inferences, for example on the role of HIV or β-glucan in the observed immune changes. Third, the heterogeneity and activity of the HIV reservoir is not fully determined by HIV CA-DNA and HIV CA-RNA measurement only, residual viremia with single copy assay could quantify recent HIV activity. Fourth, recently monocyte alterations in PLHIV were linked to cART induced oxidative stress [69]. The enrolment in our cohort was limited to virally-suppressed PLHIV on cART and, therefore, a possible independent effect of cART on immune responses could not be assessed. However, an effect of different cART regimens appears unlikely, since no differences in cytokine responses were found across the different cART regimens. Finally, due to limited inclusion of women in our cohort, generalizability to women or sex-specific analyses were not feasible.

In conclusion, we found that PLHIV on stable cART exhibit a marked and long-lasting increase in the production of IL-1β with subsequent downstream monocyte-derived cytokines, suggesting a trained immunity phenotype of monocytes. Increased translocation of β-glucan from the gastrointestinal tract was identified as a possible inducer of this innate immune phenotype. Our findings provide mechanistic insights and shed new light on the usefulness of interventions targeting the innate immune system, as well as the gut mycobiome, as an adjunctive therapy to reduce non-AIDS related co-morbidities.

Methods

Study population

A total of 211 PLHIV were recruited from the HIV clinic of the Radboud university medical center between December 2015 and February 2017 with a follow up measurement in 2018. Caucasian individuals age ≥ 18 years, on cART > 6mo with an HIV-RNA load ≤200 copies/mL and showed no signs of opportunistic infections or active hepatitis B/C were included. The control group consisted of 56 healthy individuals not using any medication at inclusion. Inclusion, sampling and sample processing of both cohorts were conducted simultaneously and uninfected controls were sampled every three months during the inclusion of PLHIV. In the follow-up measurement, we resampled 28 male PLHIV, aged >45yrs after >1yr and stratified them on the basis of their initial IL-1β production capacity in a group of low (lowest quartile) and high (highest quartile) IL-1β producers. A control group of fourteen age-sex matched healthy controls was also included. These groups were used for monocyte-only, RNA expression and β-glucan measurements. All experiments were performed by the same personnel using the HFGP methodologies (http://www.humanfunctionalgenomics.org) [19]. General information from all participants was recorded in an electronic case report form (CastorEDC, Amsterdam, The Netherlands). Clinical data were extracted from the electronic hospital information system and the 'Stichting HIV Monitoring' registry (Amsterdam, The Netherlands).

Ex vivo PBMC and monocyte stimulation

Venous blood was collected in sterile 10 mL EDTA and 8 mL serum BD Vacutainer® tubes and processed within 1-4h. Isolation of PBMCs was performed freshly collected blood by density centrifugation over Ficoll-Paque (VWR, Amsterdam, The Netherlands) as described previously [70]. Monocytes were isolated by magnetic activated cell sorting using negative bead selection with the Pan Monocyte Isolation kit according to manufacturer's instructions (Miltenyi Biotec, Leiden, the Netherlands). Cell counts, cell purity and composition were evaluated by XN-450 hematology analyzer (Sysmex Corporation, Kobe, Japan). Fresh isolated cells (monocytes 1.10^5 cells per well, PBMCs 5.10^5 cells per well) were incubated with different bacterial, fungal, viral stimuli (Table S2) at 37°C and 5% CO_2 for either 24h or 7d. For the 7d stimulation, 10% human pooled serum was added to the wells. IL-1β, IL-6, IL-1Ra, IL-10 and TNF-α were determined in the supernatants of the 24hr PBMC or monocyte stimulation experiments, using enzyme-linked immunosorbent assays (ELISA; Duoset ELISA, R&D Systems, Minneapolis, MN, USA). IL-17, IL-22 and IFNγ were measured after 7d stimulation of PBMCs (PeliKine Compact or R&D Systems). For intracellular cytokine measurements of active IL-1β and pro IL-1β (Quantikine, R&D Systems), cell pellets were lysed using Triton X-100 (Sigma, Zwijndrecht, The Netherlands).

Measurements of plasma markers and immunophenotyping

Intestinal fatty acid binding protein (IFABP), a marker of enterocyte damlage, resistin, adiponectin, leptin, IL-18 binding protein (IL-18BP), IL-18, hsCRP, sCD14 and sCD163 were measured using ELISA (Duoset or Quantikine, R&D Systems). IL-6, TNF-α, IL-10 and IL-1Ra were measured using SimplePlex Cartridges (Protein Simple, San Jose, CA, USA). 1,3-β-D-glucan (β-glucan) levels were measured with Fungitell ((Associates of Cape Cod, Liverpool, UK) and CMV IgG by ELISA (Genway Biotech, San Diego, CA, USA). All assays were performed according to manufacturer's recommendations, and samples of the different cohorts were measured simultaneously in the same plates or SimplePlex Cartridges. Monocyte subsets

were measured in whole blood on a Navios flow cytometer (Beckman Coulter, Brea, CA, USA) as described elsewhere. Antibodies used are listed in Table S3 [22].

RNA extraction and gene expression

For qPCR analyses, total RNA was extracted from monocyte cell pellets (5.10^5 cells), collected in TRIzol (Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions. Subsequently, cDNA was synthesized using iScript reverse transcriptase according to manufacturer's instructions (Invitrogen) with 500ng RNA input in 10ul reaction mix. Relative expression of genes NLRP3, TNF, IL6, IL1B was measured using SYBR Green assays (Invitrogen, Carlsbad, CA, USA) on an Applied Biosciences Step-one PLUS qPCR machine (Thermo Fischer Scientific, Carlsbad, CA, USA). Reaction volumes of 10 µL with 2 µL cDNA input. Normalization was performed using ΔΔCq based on two *(18S* and *B2M)* reference genes which were stable after stimulation. Cycling conditions included: 95°C for 10mins, [95°C for 15 seconds, 60°C for 1min seconds] (40 cycles), followed by dissociation curve analysis. Primers were designed to be intron-spanning and are listed in *table S4*.

For transcriptome analysis by RNAseq, total RNA was extracted from monocytes (5.10^5) using the QIAGEN RNeasy RNA extraction kit (Qiagen, Venlo, The Netherlands), using on-column DNase treatment. Next, ribosomal RNA removal and library preparation for next generation RNA-sequencing was achieved utilizing KAPA RNA HyperPrep Kit with RiboErase (Roche, Basel, Switzerland), following the manufacturer's protocol. The integrity and quality of prepared libraries were assessed using Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). Sequencing was performed using Illumina NextSeq 500 machine (San Diego, CA, USA) in a paired end sequencing fashion. Sequencing reads obtained from RNA-seq measurement were aligned to the hg38 human genome reference using STAR [71]. A lower cutoff of average 50 reads among all samples was used to designate a gene as being expressed in our cohort. R software and DESeq2 differential analysis package [72] were used to normalize and assess differentially expressed genes, utilizing FDR 5% and log2(Fold change) > 1 as the significance cutoff. The network of genes connected to their corresponding gene ontology was generated using cytoscape [73]. The processed RNA-seq data including normalized read counts can be accessed via GEO (GSE160184).

HIV Reservoir quantification

HIV-1 CA-DNA and CA-RNA were measured in triplicate by droplet digital PCR (ddPCR QX200 – Bio-Rad, Hercules, CA, USA) (Table S3) in CD4+ T cells isolated using EasySep Human CD4+ T Cell Isolation Kit (Stemcell technologies, Vancouver, Canada) as described previously [74]. Briefly, genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen) according to the manufacturer's protocol with an additional step of adding 75µl elution buffer on the column heated at 56°C for 10 min. CA-RNA was extracted using the Innuprep RNA kit (Westburg, Leusden, The Netherlands) with 30µl elution buffer. Total RNA was reversely transcribed to cDNA by qScript cDNA SuperMix according to manufacturer's protocol (Quantabio, Beverly, MA, USA). Before PCR amplification, genomic DNA was restricted by EcoRI (Promega, Madison, WI, USA) and cycling conditions were implemented as described previously [74]. Total HIV-1 DNA measurements were normalized by measuring the reference gene RPP30 (Table S4) in duplicate by ddPCR and expressed per million PBMCs. CA-RNA was normalized using three reference genes per patient, (B2M, ACTB and GADPH), which were measured with LightCycler 480 SYBR Green I Master mix (Bio-Rad). HIV-1 RNA copies were divided by the geometric mean of the reference genes and expressed per million PBMCs. Droplet classification and absolute quantification was performed using the ddpcRquant analysis tool with standard settings [75].

Statistics

Depending on normality, log- or inverse rank-based transformation was applied. Values outside the assay quantification limits were imputed at the respective limit. Parameters for which one of the detection limits contained > 50% of the measurements were excluded. Comparisons in baseline characteristics between groups were made using Student's T-test or Mann-Whitney U test depending on data distribution. Differences in non-continuous data were analyzed by the Pearson's Chi-square test or Fisher's exact test in case of expected counts less than five. Data were analyzed using a linear regression model. The crude model included an adjustment for sampling time and the primary analyses included age, sex and seasonality as co-variates. P-values less than 0.05 were considered statistically significant and correction for multiple testing was applied using the Benjamini-Hochberg method (FDR-correction). Data were analyzed using R (R Core Team, 2015).

Study approval

The study protocol was approved by the Medical Ethical Review Committee region Arnhem-Nijmegen (CMO2012-550) and experiments were conducted in accordance with the principles of the Declaration of Helsinki. Written informed consent was obtained from all participants.

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Supplemental table S1. Baseline characteristics validation cohort.

Characteristic	HIV (n=28)	HC (n=14)
Male sex, no. (%)	28 (100)	14 (100)
Age, median (IQR) years	56.3 (9.6)	64.3 (12.5)
cART regimen, no. (%)		
NNRTI	6 (21.4)	-
PI	2 (7.1)	-
INSTI	20 (71.4)	-
DTG	16 (57.1)	-
ELV	1 (3.6)	-
RAL	3 (10.7)	-

cART: combination antiretroviral therapy. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor. INSTI: integrase inhibitor. DTG: dolutegravir. EVG: elvitegravir. RAL: raltegravir.

Supplemental table S2. Stimulations scheme for the ex vivo cytokine production assays.

Stimulus	Final Concentration	Strain/Manufacture
	Stimulation of PBMCs for 24 hours	
LPS	1 and 100 ng/mL	E.coli/Sigma
Pam3Cys	10 µg/mL	EMC microcollections
RPMI + serum	10% human serum	Life Technologies
Poly I:C	100 µg/mL	Sas Invivogen
Imiquimod (TLR7 ligand)	5 ug/mL	Invivogen
Influenza + serum	1.25 x 10^6 PFU/mL + 10% human serum	H1N1 (in house)
Mycobacterium tuberculosis	1 µg/mL	H37Rv (in house)
E.Coli	1 x 10^6/mL	ATCC 35218 (in house)
Candida albicans conidiae	1 x 10^6/mL	UC820 (in house)
Candida albicans hyphae	1 x 10^6/mL	UC820 (in house)
Staphylococcus aureus	1 x 10^6/mL	ATCC 29213 (in house)
Cryptococcus gattii + serum	1 x 10^7/mL + 10% human serum	A1M-R265, AFLP type 6 (clinical isolate)
Streptococcus pneumonia	1 x 10^7/mL	TIGR4
	Stimulation of PBMCs for 7 days*	
RPMI + serum	10% human serum	Life technologies
Staphylococcus aureus	1 x 10^6/mL	
Cryptococcus gattii	1 x 10^7/mL	A1M-R265, AFLP type 6 (clinical isolate)
Candida albicans conidiae	1 x 10^6/mL	UC820 (in house)
Candida albicans hyphae	1 x 10^6/mL	UC820 (in house)
Streptococcus pneumonia	1 x 10^6/mL	TIGR4
Mycobacterium tuberculosis	1 µg/mL	H37Rv (in house)
Imiquimod (TLR7 ligand)	2.5 ug/mL	Invivogen

PBMCs: peripheral blood mononuclear cells; LPS: lipopolysaccharide; TLR: Toll-like receptor.
*All 7-day PBMC stimulation experiments were supplemented with 10% human pool serum

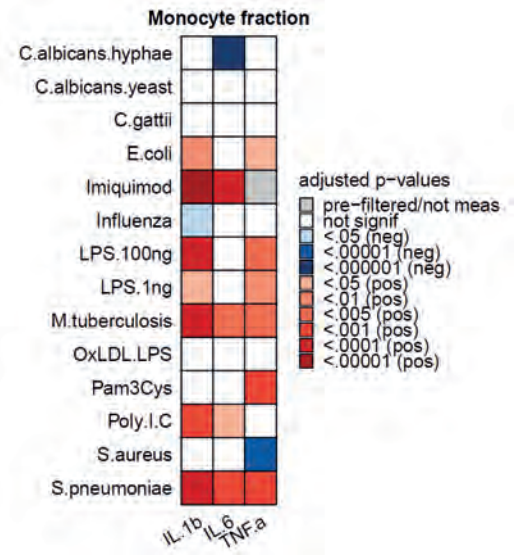
Supplemental table S3. Antibodies for immunophenotyping.

Fluorochrome	FITC	PE	ECD	PE-Cy5.5	PC7	APC	APC-AF700	APC-AF750	PB	KO
mAb	CD16	HLA-DR	CD14	CD4	CD25	CD56	CD8	CD19	CD3	CD45
Distributor	BC	BC	BC	BC	BD	BC	BC	BC	BC	BC

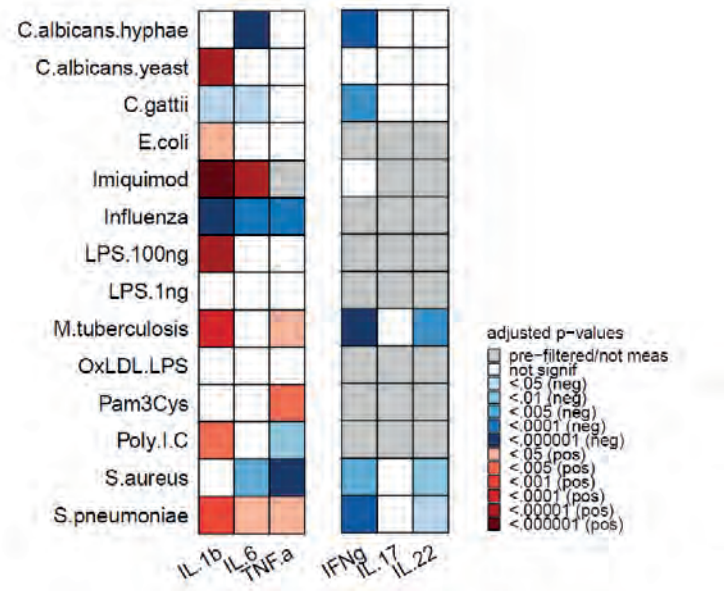
10-color flow cytometry panel. Samples were analyzed by a 3-laser Navios (BC).mAb: monoclonal antibody; BC: Beckman Coulter, CA, USA, BD: Becton Dickinson biosciences, CA, USA

Supplemental table S4. Primers and probes.

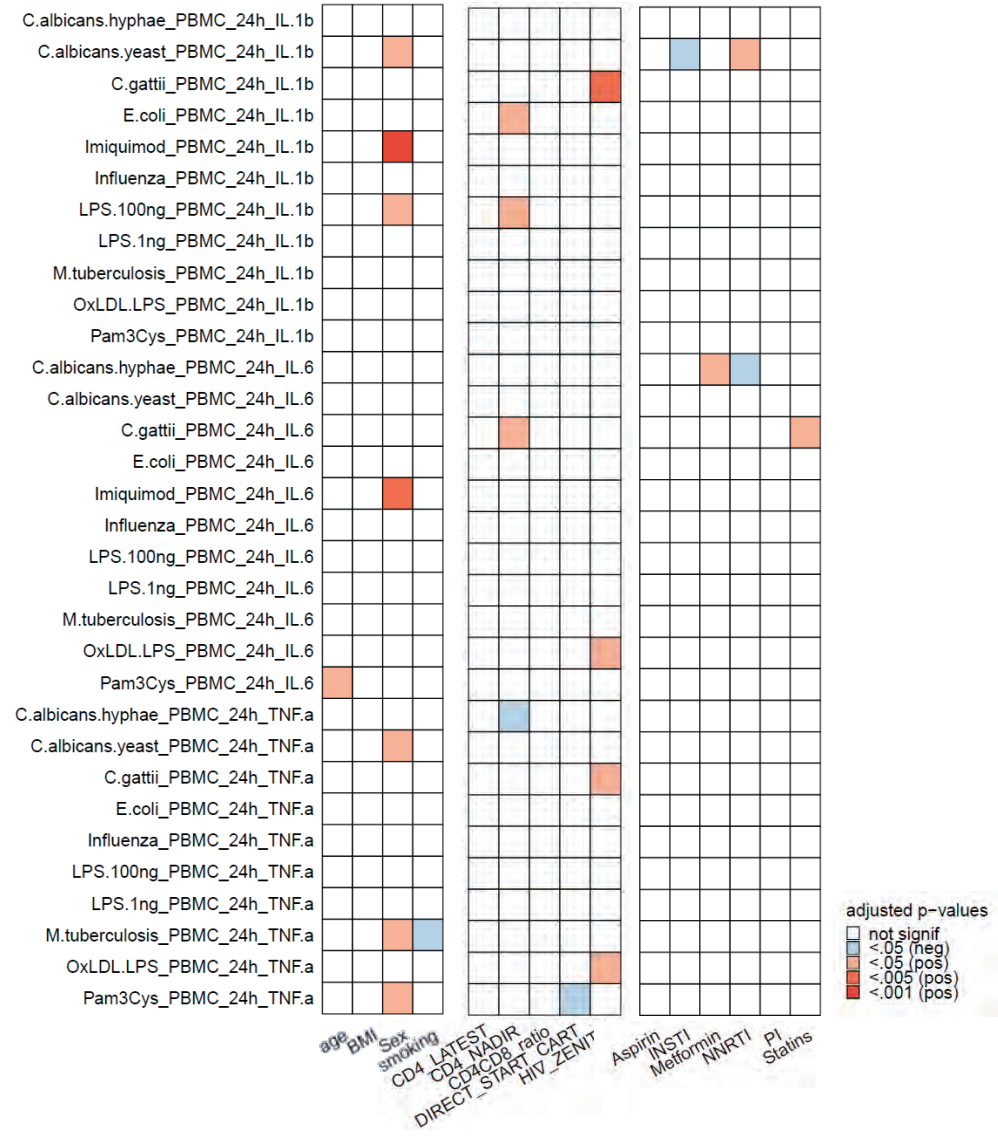
Gene	Fw/Rv/Pr	Sequence (5>3)
NLRP3	forward	GATCTTCGCTGCGATCAACA
	reverse	GGGATTCGAAACACGTGCATTA
18S	forward	GATGGGCGGCGGAAAATAG
	reverse	GCGTGGATTCTGCATAATGGT
B2M	forward	ATGAGTATGCCTGCCGTGTG
	reverse	CCAAATGCGGCATCTTCAAAC
RPL37A	forward	ATTGAAATCAGCCAGCACG
	reverse	AGGAACCACAGTGCCAGAT
TNFa	forward	CCTGCTGCACTTTGGAGTGA
	reverse	GAGGGTTTGCTACAACATGGG
IL6	forward	GGCACTGGCAGAAAACAACC
	reverse	GCAAGTCTCCTCATTGAATCC
IL1b	forward	ACAGATGAAGTGCTCCTTCCAG
	reverse	CATGGCCACAACAACTGACG
HIV-1 DNA	forward	GCCTCAATAAAGCTTGCC
	reverse	GGCGCCACTGCTAGAGATTTT
	probe	AAGTRGTGTGTGCCC
RPP30	forward	AGATTTGGACCTGCGAGCG
	reverse	GAGCGGCTGTCTCCACAAGT
	probe	TTCTGACCTGAAGGCTCTGCGCG



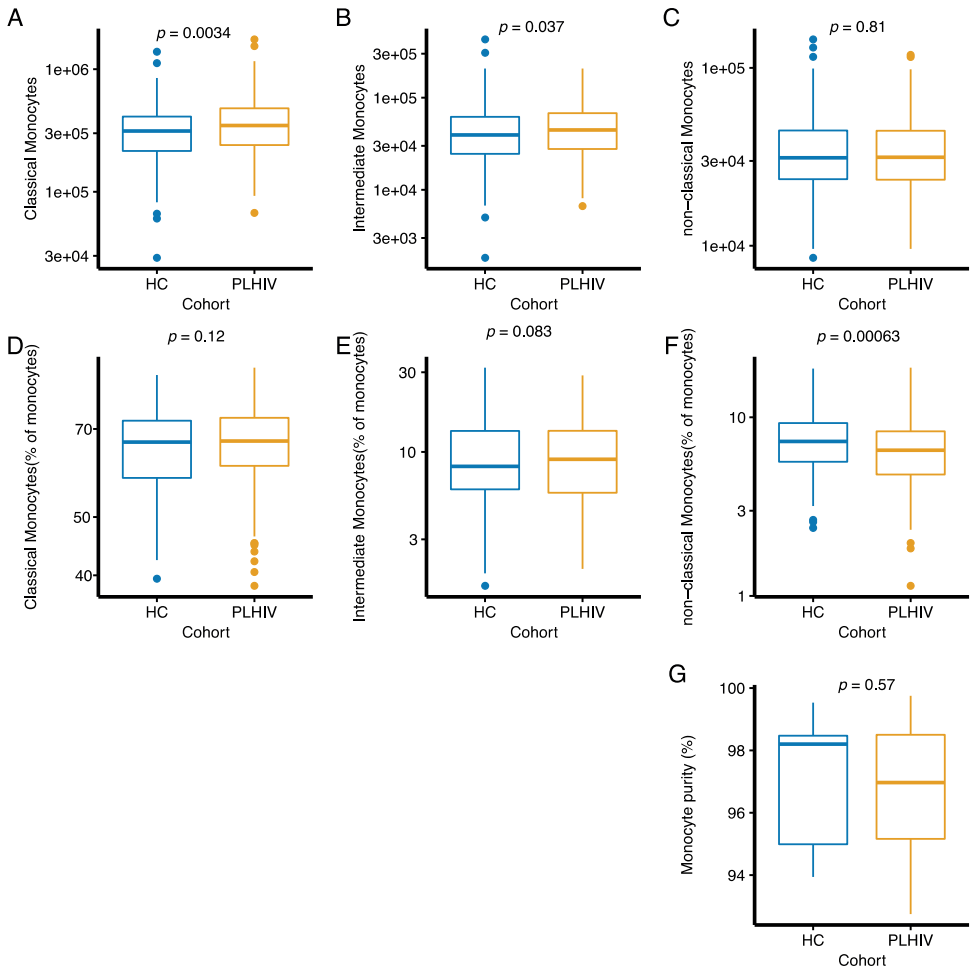
Supplemental figure S1. Adjusted P-value are depicted from a corrected model including age, sex, seasonality and monocyte fraction (of PMBCs) as covariates. Red depicts significantly higher in PLHIV, blue depicts lower in PLHIV compared to controls. All p-values are FDR corrected.



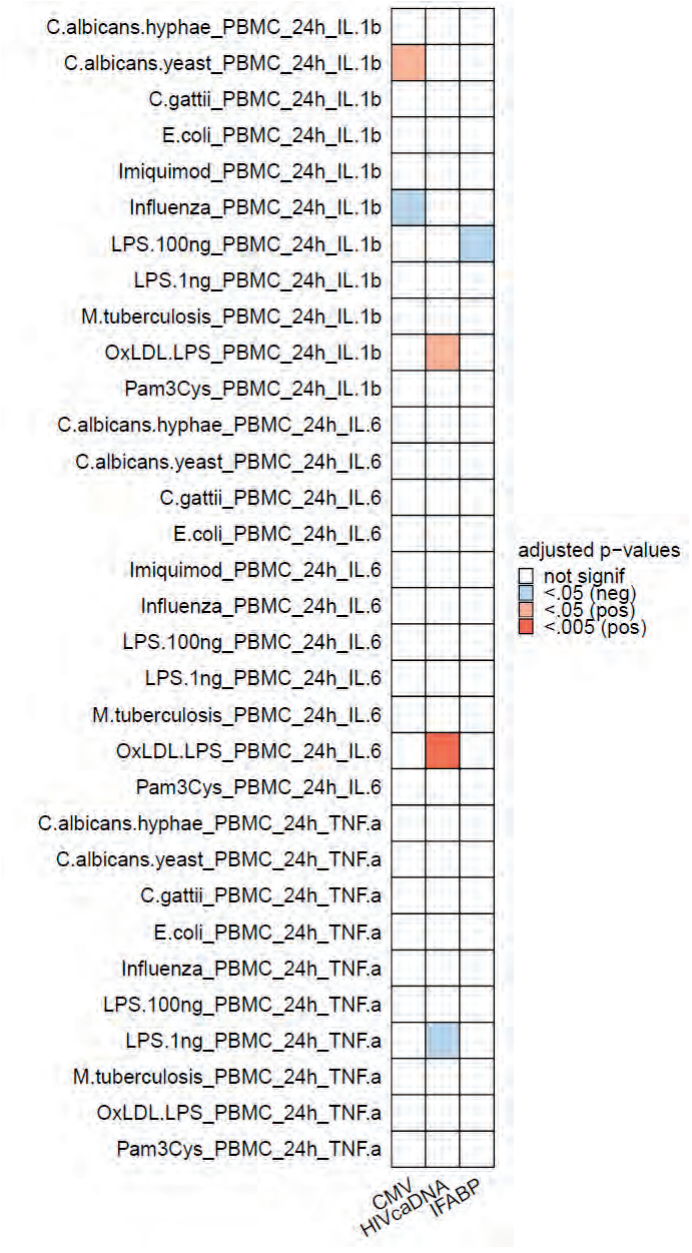
Supplemental Figure S2. Crude values 24h and 7 days cytokine production. Circulating factors in PLHIV and uninfected controls. Crude model is linear regression after inverse rank-based transformation. Corrected model included age, sex, and seasonality as covariates. Red depicts the marker is significantly increased in PLHIV; blue depicts the marker is decreased in PLHIV compared to controls. All p-values are FDR corrected.



Supplemental figure S3. Adjusted P-value are depicted from a corrected linear regression model including age, sex, seasonality as covariates. Red depicts a significant positive correlation; blue depicts negative correlation. All p-values are FDR corrected



Supplemental figure S4. Monocytes. (**A-C**) Circulating monocyte subset concentrations determined by immunophenotyping. Classical monocytes (**A**): CD14++CD16-, Intermediate(**B**): CD14++CD16+, non-classical(**C**): CD14+CD16+. (**D-F**) subsets are depicted as % of total monocytes. (**G**) Monocyte purity after CD14-negative selection magnetic bead isolation determined by Sysmex XN-450 hematology analyzer.



Supplemental figure S5. HIV reservoir, CMV seropositivity and microbial integrity. P-value are depicted from linear regression. Red depicts a significant positive correlation; blue depicts negative correlation. All p-values are FDR corrected.

Chapter 3

The architecture of circulating immune cells is dysregulated in people living with HIV on long term antiretroviral treatment and relates with markers of the HIV-1 reservoir, cytomegalovirus, and microbial translocation

Lisa Van de Wijer#, Wouter van der Heijden#, Rob ter Horst, Martin Jaeger, Wim Trypsteen, Sofie Rutsaert, Bram van Cranenbroek, Esther van Rijssen, Irma Joosten, Leo Joosten, Linos Vandekerckhove, Till Schoofs, Jan van Lunzen, Mihai Netea, André van der Ven, Hans Koenen, Quirijn de Mast

 # Equal contribution

Frontiers in immunology 2021; 12: 661990.

Abstract

Despite adequate combination antiretroviral therapy (cART), the immune system in people living with HIV (PLHIV) only partially recovers, predisposing them to non-AIDS-related comorbidities and impaired vaccination responses. Here, we compared the circulating white blood cell (WBC) architecture between long-term treated people living with HIV (PLHIV) and healthy controls, by characterizing 108 peripheral WBC subsets by flow cytometry. We next assessed the relation between WBC subsets and clinical factors, markers of the HIV-1 reservoir (CD4-cell-associated HIV-1 DNA [CA-DNA] and CA-RNA), CMV IgG titers, circulating inflammation markers, and immunoglobulins. Lastly, we assessed lymphocyte function by measuring *ex vivo* cytokine production capacity of interleukin (IL)-17, IL-22, and interferon (IFN)-ɣ in response to different bacterial, fungal and viral stimuli. We included a total of 211 virally suppressed adult PLHIV (n=211) on combination antiretroviral therapy (cART) for a median (IQR) of 6.6 (7.7) years and 56 healthy controls. PLHIV showed a loss of naïve T cells, an increase of T helper (Th) 17 cells and effector T cells, as well as dysregulated B cell memory responses. Th17 percentages were related with inflammation markers (IL-6 and soluble sCD14), gut homing chemokines (CCL20) and intestinal fatty acid binding protein (IFABP). In the innate arm, PLHIV exhibited an expansion of monocytes and a clear loss of natural killer (NK) cells. Levels of CA-DNA and CA-RNA were associated with higher percentages of central memory CD4+ cells, Th17, and regulatory T cells, whereas CMV seropositivity was associated with an expansion of effector T cells in PLHIV. Functionally, IL-22 and IL-17 responses did not differ between PLHIV and controls, whereas IFN-ɣ production was reduced in PLHIV, especially in those with higher CA-DNA levels. Together, our data show a generalized dysregulated pro-inflammatory circulating immune profile in long-term treated PLHIV. These changes in WBC cells related with markers the HIV-1 reservoir and translated into reduced IFN-ɣ responses, reinforcing the need for early cART initiation.

Introduction

Combination antiretroviral therapy (cART) has drastically curtailed morbidity and mortality in people living with HIV (PLHIV)[1]. Still, PLHIV remain at an increased risk for pneumococcal infections, *Mycobacterium tuberculosis* (*M. tuberculosis*) reactivation and impaired vaccine responses[2-7]. Moreover, HIV infection predisposes to non-infectious comorbidities, such as cardiovascular disease and non-AIDS-related cancer[8], which share an underlying pathophysiological pathway characterized by a persisting and inappropriate activation of innate and adaptive immune cells[9]. Together, these observations point towards a disbalance in the homeostasis of the immune system, characterized by immunodeficiency on the one hand, and chronic inflammation on the other hand[8].

HIV-1 preferentially infects and kills activated CD4+ T cells, leading to rapid and severe CD4+ T cell depletion in the gut and increased microbial translocation[10]. A small proportion of these cells remains latently infected with replication competent virus and defective proviruses, called the HIV-1 reservoir[11]. The HIV-1 reservoir and increased microbial translocation, together with lifestyle factors and co-infections such as cytomegalovirus (CMV), are considered important drivers of the disrupted immune homeostasis in PLHIV[11-15]. However, published data have shown inconsistent or even contradictory findings. Heterogeneity in study populations, limited sample sizes of study populations and differences in lifestyle factors, including use of tobacco and recreational drugs, may underlie these inconsistencies and emphasize the need for an integrative approach in evaluating the immune system in PLHIV on chronic cART.

The Human Functional Genomics Project (HFGP) aims to disentangle variation in the immune system in different cohorts of healthy individuals and individuals with underlying conditions. It combines multiple levels of analyses and data integration, including demographic and lifestyle data, data from 'omics technologies', en functional immune data[16]. As part of this project, we previously identified relevant environmental and host factors for circulating white blood cell (WBC) populations in healthy individuals[17]. Embedded within the HFGP, we established a cohort of 211 virally suppressed PLHIV (200HIV) and showed that these individuals exhibited a sustained pro-inflammatory immune phenotype with priming of the interleukin (IL)-1β pathway[18]. In the present study, we used the same cohort to comprehensively assess the peripheral WBC composition during chronic treated HIV infection with a special focus on the adaptive immune system. We compared their WBC populations with those of healthy individuals and assessed associations with demographic and lifestyle factors, different HIV-specific factors, circulating cytokines, as well as *ex vivo* cytokine production capacity of peripheral blood mononuclear cells (PBMCs) after stimulation with different bacterial, fungal and viral antigens.

Methods

Study population

This study is part of the HFGP (www.humanfunctionalgenomics.org)[16]. Between 14 December 2015 and 6 February 2017, individuals living with HIV were recruited from the HIV clinic of Radboud university medical center. Inclusion criteria were: Caucasian ethnicity, age ≥ 18 years, receiving cART > 6 months, and latest HIV-RNA levels ≤200 copies/ml. Exclusion criteria were: signs of acute or opportunistic infections, antibiotic use <1 month prior to study visit, or active hepatitis B/C. The control population consisted of 56 healthy adult individuals (56P cohort) who were longitudinally sampled four times in three-month intervals. Inclusion, sampling and sample processing of both cohorts were conducted simultaneously and by the same personnel. The 56P participants were selected as a subset of a larger cohort of 534 healthy individuals (500FG) which was phenotypically assessed two years earlier according to the same methods[17,19]. Given the larger sample size, differences in cell-cell associations were compared between 200HIV and the 500FG.[17] General information from all participants was recorded in the Castor Electronic Data Capture program (Castor EDC, CIWIT B.V., Amsterdam, The Netherlands), using questionnaires on socio-demographic information, lifestyle and life-events. Clinical data were extracted from medical files and the 'Stichting HIV Monitoring' registry (Amsterdam, The Netherlands).

Ethics

The study protocols were approved by the Medical Ethical Review Committee region Arnhem-Nijmegen (ref. 42561.091.122) and conducted in accordance with the principles of the Declaration of Helsinki. All study participants provided written informed consent.

Cell processing

Venous blood was collected in sterile 10 ml EDTA BD Vacutainer® tubes between 8 and 11 am and processed within 1-4 hours. Cell counts were determined using a Sysmex XN-450 automated hematology analyzer (Sysmex Corporation, Kobe, Japan). Erythrocyte-lysed whole blood samples were obtained after 10 minutes incubation of 1.5 ml EDTA-anticoagulated blood with lysis buffer containing 3.0 M NH_4Cl, 0.2 M $KHCO_3$ and 2 mM Na_4EDTA. The remaining WBC were washed twice, by adding 25 ml phosphate-buffered saline 1x (PBS, Braun, Melsungen, Germany) and centrifuging at 452 x g for 5 min at room temperature. Before staining, cells were resuspended in 300 µl of PBS enriched with 0.2% bovine serum albumin (BSA, Sigma-Aldrich, Zwijndrecht, Netherlands). Isolation of PBMCs was performed by density centrifugation of EDTA-anticoagulated blood diluted 1:1 in pyrogen-free PBS over Ficoll-Paque (VWR, Amsterdam, The Netherlands) as described previously[20]. Cells were adjusted to 5.0 x 10^6 cells/ml.

Flow cytometry

Supplementary Table 1 summarizes the antibody clones and the fluorochrome conjugates used for the different panel fluorescent staining mixes. Staining was performed on 100 µl/well erythrocyte-lysed blood (panel 1-3,5) or 0.5 x 10^6 cells/well freshly isolated PBMCs (panel 4). Cells were stained according to previously described procedures (see also Supplementary Methods)[17]. Samples were measured on a 10-color Navios flow cytometer (Beckman Coulter, Fullerton, CA, USA), equipped with three solid-state lasers (488 nm, 638 nm, and 405 nm) [17]. Gating was conducted manually and verified by two independent specialists to prevent gating errors. Samples were analyzed within 4-5 hr after blood collection, using five distinct and complementary 10-color antibody panels: 1. general; 2. T cell; 3. B cell; 4. intracellular T cell/Treg; 5. Chemokine receptors. Staining and gating strategies are summarized can be found in Supplementary Figure 1. Flow cytometry data were analyzed using Kaluza software version 1.3.

PBMC stimulation experiments

Freshly isolated PBMCs were incubated with different stimuli (Supplementary Table 2) including bacterial (*Staphylococcus aureus*, *M. tuberculosis*, *Streptococcus pneumoniae [S. pneumoniae]*), fungal (*Cryptococcus gattii*, *Candida albicans [C. albicans]* hyphae and yeast) and other relevant antigens (Imiquimod, TLR7 ligand), in round-bottom 96-well plates (Greiner Bio-One, Frickenhausen, Germany) with 0.5 x 10^6 cells/well at 37°C and 5% CO_2 in the presence of 10% human pooled serum for seven days. Supernatants were stored at -20°C. Levels of the lymphocyte-derived cytokines IL-17, IL-22, and interferon (IFN)-γ were measured in the supernatants (PeliKine Compact or Duoset ELISA, R&D Systems).

Plasma markers

Serum levels of immunoglobulin (Ig)M and IgG were measured by immunonephelometry using a Beckman Coulter Imager and Beckman Coulter reagents. Measurements were standardized using certified European reference material 470 (ERM®-DA470). CMV IgG antibodies were measured in serum using ELISA (Genway Biotech Inc., San Diego, CA, USA) according to the manufacturer's instructions. Markers of systemic inflammation, high-sensitive C-reactive protein (hsCRP) and soluble CD14 (sCD14), and microbial translocation, intestinal fatty acid binding protein (IFABP), were measured by ELISA (Quantikine, R&D Systems) according to the manufacturer's instructions. IL-6 was measured using a SimplePlex Cartridge (Protein Simple, San Jose, CA, USA). Circulating plasma CCL20, IL-17A, and IFN-γ were measured using the commercially available Olink Proteomics AB Inflammation Panel as described previously[21,22]. In this Proceek Multiplex proximity extension assay, proteins are recognized by pairs of antibodies coupled to cDNA strands, which bind when they are in close proximity and extend by a polymerase reaction. Detected proteins were normalized according to interplate controls and measured on a log2 scale as normalized protein expression values.

Cell-associated HIV-1 DNA and cell-associated HIV-1 RNA quantification in CD4+ T cells

The HIV reservoir was assessed by analyzing CD4+ cell-associated HIV-1 DNA (CA-DNA) and CA-RNA. In virally suppressed patients, the CA-DNA roughly equals the integrated HIV-1 DNA, being replication competent or not[23], while CA-RNA is associated with recent HIV-1 transcriptional activity and serves as a proxy for the active proviral reservoir[24]. CA-DNA and CA-RNA were measured in triplicate by droplet digital PCR (ddPCR – Bio-Rad) in CD4+ T cells isolated using EasySep Human CD4+ T Cell Isolation Kit (Stemcell technologies, Vancouver, Canada) as described previously[25]. CA-DNA was extracted by the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with the addition of 75µl elution buffer on the column heated at 56°C for 10 minutes. CA-RNA was extracted using the Innuprep RNA kit (Westburg, Leusden, The Netherlands) with 30µl elution buffer. Total RNA was reversely transcribed to cDNA by qScript cDNA SuperMix according to manufacturer's protocol (Quantabio, Beverly, MA, USA). CA-DNA was normalized by measuring the reference gene RPP30 (Supplementary Table 3 and Supplementary Methods) in duplicate by ddPCR and expressed per million PBMCs. CA-RNA was normalized using three reference genes per patient, (B2M, ACTB and GADPH), which were measured with LightCycler 480 SYBR Green I Master mix. HIV-1 RNA copies were divided by the geometric mean of the reference genes and expressed per million PBMCs. ddPCR data analysis was performed using the ddpcRquant tool with standard settings for thresholding and absolute quantification[26].

Statistical analyses

A detailed description of the statistical methods can be found in the Supplementary Methods. Given the impact of cohort differences in the frequency of main WBC types (e.g. CD4+) on the frequencies of their subsets (e.g. CD4+ regulatory T cells [Tregs]), results were primarily reported as WBC percentages, unless stated otherwise. WBC percentages were calculated by dividing the cell count of each subpopulation by its respective parent (one level up) or, where relevant, grandparent (two levels up; Supplementary Table 4). Data curation of cytokine, soluble marker, and immunoglobulin data was done according to previous methods[19]. For comparisons between cohorts, we used all four longitudinally collected data points from the 56P cohort as independent measurements. Because of the known seasonality effects on WBC, this approach was considered preferable over selecting one of the data collection points or summarizing the data points. Data were normalized using an inverse rank transformation algorithm.

Comparisons in baseline characteristics between groups were made using Student's t-test (or Mann-Whitney U test) for continuous variables, and Pearson's Chi-square test (or Fisher's exact) for categorical variables. Linear regression was used to compare WBC between cohorts, and to calculate associations between WBC and clinical and virological factors. All analyses were corrected for age, sex, time since January 2015 and seasonal effects. Cytokine analyses between cohorts were also corrected for CD4+ and CD8+ T cell percentages. Spearman correlations were used as the distance metric for unsupervised hierarchical WBC count clustering. Two-sided FDR-corrected p-values < 0.05 were considered statistically significant[27]. Effect sizes are reported as Spearman's Rho or standardized beta coefficients (β). Data were analyzed using the statistical programming language R (version 3.4.3, R Core Team, 2012).

Results

General and HIV-specific characteristics

Data from 211 PLHIV and 56 controls were analyzed in this study (Table 1). PLHIV were older (median [IQR] 52.5 [13.2] vs 30.0 [27.1] years, $p<0.0001$) and more often male (193/211 [91.5%] vs 34/56 [60.7%], $p<0.0001$) than controls. PLHIV had received cART for a median of 6.6 (7.7) years and 205/210 (98%) had plasma HIV-1 RNA <50 copies/mL at time of study visit. Analyzing the HIV reservoir, we found detectable levels of total CA-DNA in 207/208 (99.5%) and CA-RNA in 210/210 (100%); CA-DNA and CA-RNA levels were highly correlated (Supplementary Figure 2A; $R=0.68$, $p<2.2\cdot10^{-16}$). In general, PLHIV with higher CA-DNA and CA-RNA levels were older, had been living with and treated for HIV for a longer period, and had lower nadir CD4+ T cell counts (Supplementary Figure 2A). We observed no significant differences in CA-RNA and CA-DNA between PLHIV with plasma HIV-1 RNA <50 copies/mL (205/210 [98%]) and those with plasma HIV-1 RNA 50-200 copies/mL (5/210 [2%]; Supplementary Figure 2B). PLHIV with at least once a viral load of >50 copies/mL during year prior to study visit (23/210 [11.0%]) had higher levels of CA-RNA (p=0.0077), but no differences in CA-DNA (p=0.076; Supplementary Figure 2B).

Alterations in WBC composition and cell-cell associations in chronic HIV

We analyzed 108 WBC populations, including 93/107 (87%) B and T cell subsets and 14/107 (13%) innate cell subsets (neutrophils, monocytes, natural killer [NK] and natural killer T cells [NKT]). Figure 1, Supplementary Figure 3 and Table 2 show the differences in WBC composition between PLHIV and controls.

Principal component analysis (PCA) of all WBC populations showed clear differences in clustering between PLHIV and controls (Figure 1A). As expected, PLHIV had an expansion of CD8+ cells and a reduction of CD4+ T cells (Table 2). Functionally, CD4+ T cells comprise a diverse population of cells including T helper (Th) cells, which fulfill essential roles for viral (Th1, CCR6-CXCR3+CCR4-), parasitic (Th2, CCR6-CXCR3-CCR4+), and mucosal (Th17, CCR6^{+}CXCR3-CCR4+) immunity[28], and Tregs, which are essential for controlling

inflammation[29]. Despite reduced CD4+ T cell counts, Th frequencies (CD4+ CD45RA- CD25-) were markedly increased in PLHIV compared to controls (Table 2). Within the Th pool, Th2 percentages were reduced in PLHIV compared to controls, whereas Th1 percentages did not differ. Remarkably, Th17 percentages and frequencies were increased in PLHIV (Figure 1B, Table 2). While Treg percentages (of CD4+ cells), including highly suppressive Tregs co-expressing the transcription factors FoxP3 and Helios, were also increased in PLHIV, Treg frequencies were reduced,(Figure 1B, Table 2), suggesting that this relative increase of Tregs mainly results from a loss of other CD4+ subsets[30]. Among Tregs, we found no differences in the percentage of activated (HLA-DR+) and effector Tregs (CD45RA-).

The relationship between pro-inflammatory Th17 cells and Treg must remain balanced to preserve functional immunity. Altered ratios have been described in untreated HIV (lower Th17/Treg), autoimmune disease and cancer (higher Th17/Treg)[10,31,32]. Here, we found increased Th17/Treg ratios among virally suppressed PLHIV, irrespective of the Treg identification marker used (Figure 1B and Supplementary Figure 4). Furthermore, out of the Treg population, the percentage of Th17-like CCR6+ Tregs was increased in PLHIV, further contributing to a pro-inflammatory state. Developmentally, T cells evolve from naive T cells to antigen experienced central memory (CM), effector memory (EM), and effector cells[33,34]. HIV not only differentially affects functional subpopulations, but also disrupts these developmental stages. While naïve $CD4^+$ and $CD8^+$ T cells were reduced, memory and effector cells were expanded in PLHIV (Figure 1B, Table 2). Likewise, we found higher percentages of proliferating (Ki67+) CD4+ and CD8+ T cells in PLHIV, indicating increased cell turnover. These results suggest a shift from naive cells towards terminally differentiated cells in HIV, even if viral replication is under control[35], which cannot be explained by age, sex, or season as we corrected our models for these factors.

Changes in the B cell compartment have also been described in PLHIV, including loss of CD27+ memory B cells[36,37]. Furthermore, viremia and low CD4+ T cell counts have been associated with the expansion of terminally differentiated B cells and immature B cells respectively[36,37]. In our study, we observed clear differences in B cell development in PLHIV illustrated by increase of naïve B cells (IgD+IgM+CD27-) and plasmablasts (IgD- IgM- CD38++) and a loss of memory B cells (IgD+IgM+CD27+), transitional B cells (CD24++CD38++) and natural effector B cells (CD24+CD38+IgD+IgM+, Figure 1B, Table 2). Adequate B cell maturation further requires optimal communication between B and T cells. We therefore sought to identify differences in WBC co-regulation between PLHIV and HIV-uninfected individuals by comparing cell-cell associations between PLHIV and participants from the 500FG cohort. Details of this 500FG cohort have been reported previously (500FG, n=534)[17]. Using the same immunophenotyping techniques, we observed weaker correlations between naïve, CM and EM CD4+ T cells and several B cell subpopulations (including class-switch memory B cells) within PLHIV than in participants of the 500FG cohort, suggesting altered B/T cell interactions (Figure 1C). These

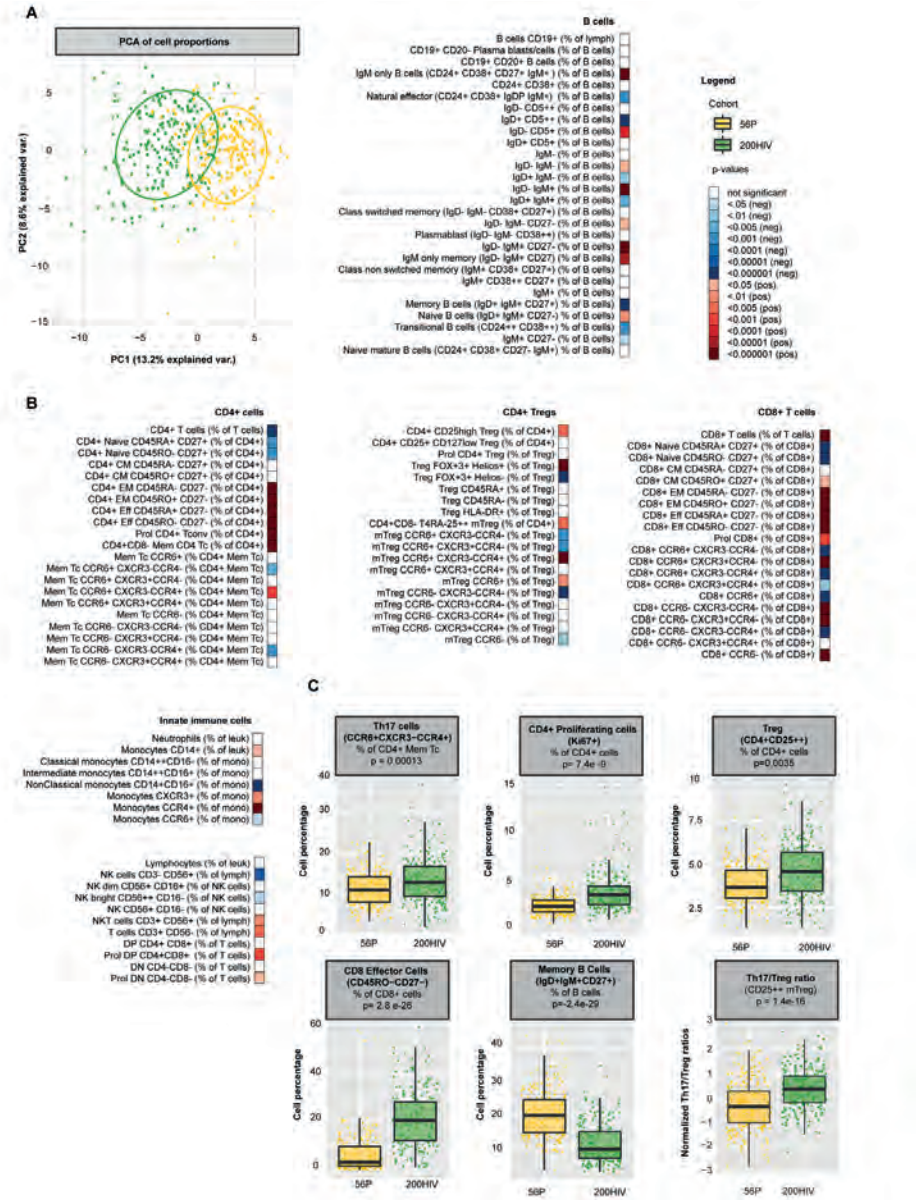


Figure 1. Differences in WBC percentages between PLIV and healthy individuals. Differences in WBC percentages (n=108 WBC subsets) between PLHIV (n=211) and healthy controls (n=56). (**A**) Principal component biplot showing standardized variance for the two principal component of all WBC subsets. The ovals represent the different cohorts. (**B**) Boxplots showing examples of cohort differences in cell percentages and Th17/Treg ratios (Treg identification marker CD25++). Inverse-rank transformed data were analyzed using linear regression and adjusted for age, sex, sampling time, and season. (**C**) Exploratory analysis depicting cell-cell associations between a total of 77 available WBC subsets that were significantly stronger (red) or weaker (blue) in PLHIV compared to healthy controls (n=534). FDR-adjusted p-values are obtained after 10 000 permutations and adjusted for age, sex, sampling time and season. HC: healthy control; PLHIV: people living with HIV; WBC: white blood cells.

differences were not attributable to the influence of age, sex, or season as these factors were regressed out of the analysis.

Apart from changes in the adaptive cell compartment, we observed clear changes in the innate WBC compartment in PLHIV reflected by an expansion of monocytes and NKT cells and a clear loss of NK cells, specifically the cytokine-producing NK bright cells (Table 2) Together, these data show a widespread functional and developmental dysregulation of the immune system in virally suppressed PLHIV. This dysregulation encompasses both the innate and the adaptive compartment and results in a pro-inflammatory immune environment with an expansion of monocytes, pro-inflammatory Th and effector T cells, and dysregulated B cell memory responses.

Age, sex, and smoking influence the WBC composition during chronic HIV infection

Apart from the effects of HIV, demographic and lifestyle-related factors may influence the composition of the circulating WBC populations. Indeed, we found that older age was associated with an increase of innate immune cells and differential effects on B and T cell populations, for example with lower percentages of naïve T cells (CD4+ T cells β=-0.29, p=0.00020; CD8+ T cells β=-0.29, p=0.00020) and B memory cells (β=-0.39, $p=1.5{\cdot}10^{-7}$) and higher percentages of memory T cells (e.g. CD8+ CM β=0.22, p=0.00064) and mature naïve B cells in PLHIV (β=0.25, p=0.00087, Figure2 and Supplementary Tables 5 and 6). Sex-dependent influences of the WBC composition, reflected by more effector and EM cells and fewer naïve (B and T) cells in males in HIV infection, resembled those observed previously observed in healthy individuals[19,38].

Lifestyle risk behaviors such as smoking (63/211 [29.9%]), heavy drinking (28/211 [13.3%]), and regular drug use (61/211 [28.9%]) were highly prevalent in PLHIV (Table 1). We found that packyears (reflecting total tobacco exposure) were associated with higher frequencies of neutrophils (β=0.22, p=0.033), Treg (β=0.22, p=0.033), CD8+ subsets (e.g. CCR6-CXCR3-CCR4+ β=0.24, p=0.025), and class switched memory B cells (β=0.20, p=0.042, Supplementary Table 6). Active smoking correlated with higher proportions of Th17 (β=0.21, p=0.031) and CCR6+ Tregs (β=0.26, p=0.0028, Figure 2, Supplementary Table 5). Neither heavy drinking, nor regular drug use affected the WBC composition.

HIV-1 reservoir and CMV affect the WBC composition in chronic HIV infection

We further assessed the effects of relevant clinical and virological factors on the WBC composition in PLHIV. First, we explored associations with the history of immune suppression and treatment-related factors. We found that nadir CD4+ cell counts were closely associated with both B and T cell percentages in PLHIV (Figure 2 and Supplementary Tables 5 and 6), *i.e.* higher counts were associated with higher percentages of naïve CD4+ T cells (β=0.45, $p=1.2{\cdot}10^{-9}$) and memory B cells (β=0.18, p=0.034). In contrast, we observed no effects of the duration of HIV infection or cART (regimen) on the WBC composition (Supplementary Tables 5 and 6).

Second, we explored the relationship with markers of the HIV reservoir. CA-DNA was negatively associated with CD4+ T cell percentages (β=-0.33, $p=9.8{\cdot}10^{-5}$) and positively with CD8+ T cell percentages (β=0.34, $p=7.0{\cdot}10^{-5}$). Within the CD4+ T cell pool, CA-DNA correlated with higher percentages of CM and Th17 cells (β=0.21, p=0.026 and β=0.24, p=0.0081 respectively; Figure 3 and Supplementary Tables 7 and 8) Higher CA-DNA was also associated with more CD4+ T cell proliferation (Ki67+, β=0.35, $p=3.3{\cdot}10^{-5}$) and Treg activation (HLA-DR+, β=0.23, p=0.0081). We found similar, generally weaker, associations between T cells and CA-RNA, but no relation with the relative HIV transcription level (CA-RNA/CA-DNA ratios; Supplementary Table 7 and 8). Lastly, we observed several associations between CA-DNA and B cells: higher CA-DNA levels correlated with reduced percentages of IgD+ only memory B cells (β=-0.27, p=0.00068) and, albeit non-significantly, with reduced percentages of natural effector B cells (CD24+CD38+IgD+IgM+, β=-0.18, p=0.056), and class unswitched memory B cells (IgD+IgM+CD27+, β=-0.18, p=0.053).

Third, like in untreated PLHIV, CMV remains a major pathogen in immune suppressed PLHIV. As CMV is known to affect WBC populations in healthy controls[39], we explored the association of CMV serostatus with WBC composition. 198 of the PLHIV (93.8%) were seropositive for CMV. In line with findings in healthy individuals[39], CMV seropositivity correlated with higher percentages of effector, EM T cells (e.g. with CD8+ effector cells β=0.43, $p=1.7{\cdot}10^{-9}$ and CD4+ EM cells β=0.30, p=0.00011; Figure 3 and Supplementary Table 7 and 8) and NKT cells (β=0.29, p=0.00029), yet with lower percentages of Th17 cells (β=-0.26, p=0.00065). B cells were not directly affected by CMV.

Microbial translocation, inflammation and Th17 differentiation in chronic HIV infection

One of our key findings is the shift of naïve cells into Th17 in PLHIV compared to healthy controls, which related with lower nadir CD4+ T cells counts and higher levels of CA-DNA. To further assess the potential underlying mechanisms, we explored the relationship between these WBC subsets, markers of chronic inflammation, and markers of microbial translocation. As discussed above, naïve T cells are able to differentiate into different Th subsets depending on the cytokine environment[40]. We previously showed that PLHIV in our cohort exhibited a pro-inflammatory profile with increased levels of hsCRP (p=0.00022) and sCD14 (a marker of monocyte activation, p=0.0025; Figure 4A) as well as markedly elevated monocyte-derived cytokine responses, particularly IL-1β [18]. Such a pro-inflammatory cytokine environment may push the differentiation of naive CD4+ cells into Th17 cells [40]. Indeed, we observed positive

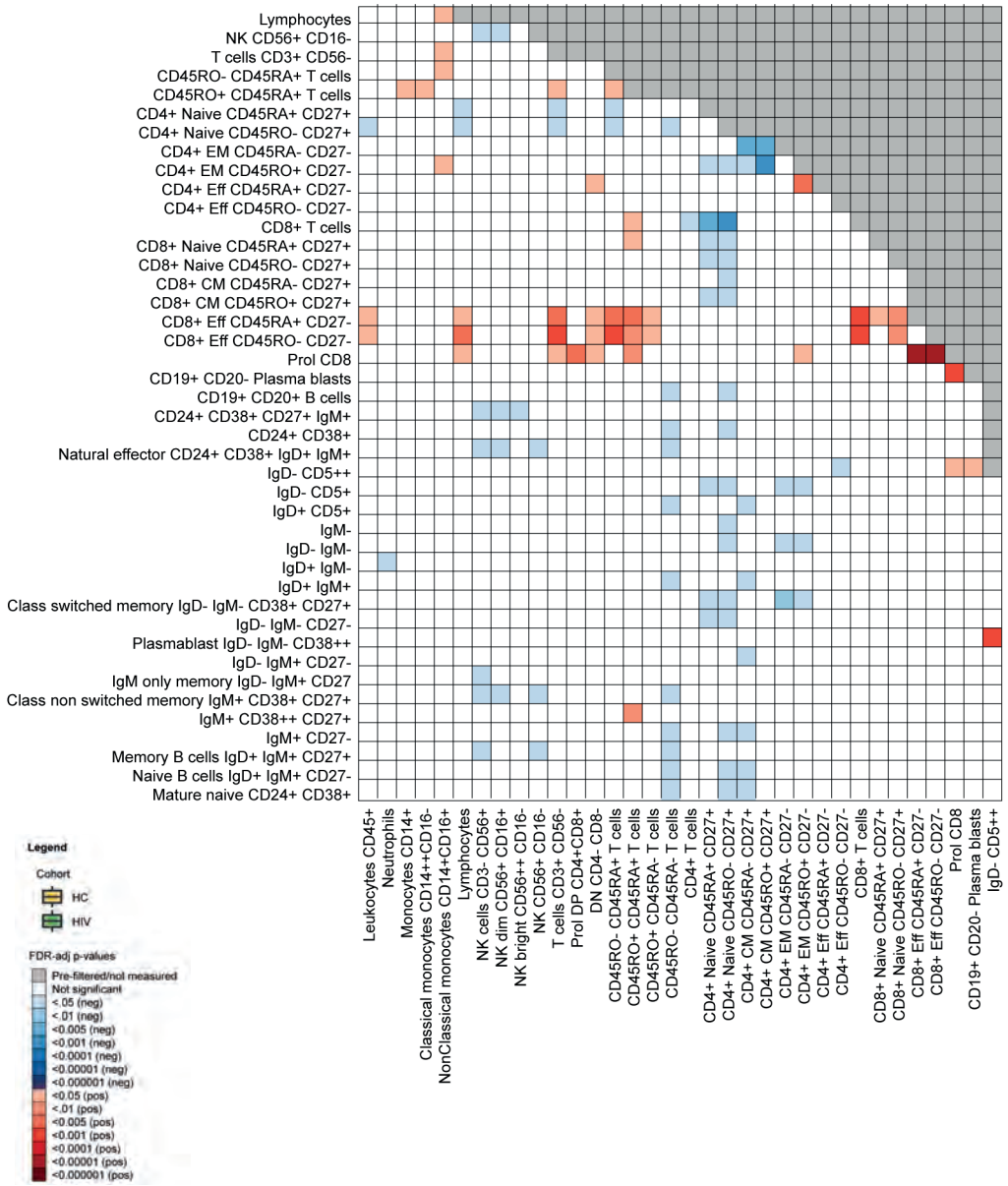


Figure 2. Clinical determinants of WBC percentages in PLHIV . (**A**) Heatmap of WBC percentages (n= n=108 WBC subsets) that were significantly associated with any of the clinical determinants tested in 211 PLHIV; WBC subsets that showed no significant correlations with any of the parameters (n=37) have been removed from the figure. (**B**) Examples of WBC subsets that were significantly associated with age, sex, smoking, or CD4 nadir. Inverse-rank transformed data were analyzed using linear regression and adjusted for age, sex, sampling time, and season. For color coding of the FDR-adjusted p-values see legend. PLHIV: people living with HIV; WBC: white blood cells.

associations between Th17 percentages and circulating IL-6 (β=0.21, p=0.014) and sCD14 (β =0.17, p=0.035; Figure 4B,C). Notably, Th17 cells fulfill an essential role in mucosal defense and gut Th17 cells are known to be severely depleted during acute HIV[10].

To test whether gut integrity might have been compromised in our cohort, we measured levels of the microbial translocation marker IFABP and found increased levels in PLHIV compared to healthy controls ($p=7.6 \cdot 10^{-5}$; Figure 4D), suggesting ongoing microbial translocation in chronic treated HIV, especially in those with higher CA-RNA and CA-DNA (CA-RNA β =0.18, p=0.0091 and CA-DNA β=0.21, p=0.0025, Figure 4E). Higher levels of IFABP were associated with an expansion of peripheral blood Th17 (β=0.17, p0.043; Figure 4B). Homing of Th17 to the gut (and other tissues, such as the skin) is directed by the chemokine CCL20, which is produced by tissue and immune cells (neutrophils and monocytes) and binds uniquely to the CCR6+ receptor[41]. Correspondingly, we found strong associations between CCL20 and Th17 percentages (β=0.22, p=0.0037), CCL20 and Th17/Treg (β=0.26, p=0.00081 for Th17/FoxP3+ Treg, β=0.27, p=0.00081 for Th17/CD127low Treg, and β=0.19, p=0.013 for Th17/CD25++ Treg), CCL20 and circulating IL-17A levels (β=0.47, $p=1.2 \cdot 10^{-11}$) and CCL20 and IFABP (β=0.24, p=0.0011; Figure 4F) . Together, these results suggest that the pro-inflammatory environment in chronic HIV may promote the differentiation of circulating naïve CD4+ T cells into Th17 cells and that these changes may be associated with changes in gut permeability and gut homing.

Functional consequences of changes in adaptive immune cells in chronic HIV

As our results indicated significant changes in circulating immune cell populations in PLHIV, we next analyzed the possible functional consequences. First, we measured the cytokine production capacity of PBMCs after *ex vivo* stimulation with different stimuli. We found strong correlations between NK and T-cell percentages and IFN-γ, IL-17, and IL-22 production capacity (Figure 5A and Supplementary Tables 9 and 10): IFN-γ responses correlated with percentages of NK dim, CD4+ and CD8+ EM cells, whereas IL-22 responses correlated with CCR6+ CM CD4+ cell percentages. As expected, Th17 percentages were associated with increased circulating IL-17A (β=0.16, p=0.029) and *ex vivo* IL-17 responses to *C. albicans* (β=0.19, p=0.047) and with reduced IFN-γ responses (Figure 5A, E and Supplementary Tables 9) Despite the expansion of Th17 cells among PLHIV compared to controls, *ex vivo* production capacity of IL-17 or IL-22 did not differ, suggesting that the functional capacity of these cells may be compromised. In contrast, IFN-γ production upon stimulation with *C. albicans* hyphae (p=0.012) and *M. Tuberculosis* (p=0.0025) was reduced in PLHIV (Figure 5B). Among PLHIV, lower *ex vivo* IFN-γ production to stimulation with Imiquimod ((β =-0.21, p=0.0020) and *S. pneumoniae* (β=-0.22, p=0.0011) and lower circulating IFN-γ concentrations (β=-0.18, p=0.014) were associated with higher CA-DNA levels (Figure 5C, D). No associations were found between *ex vivo* cytokine production and CMV seropositivity.

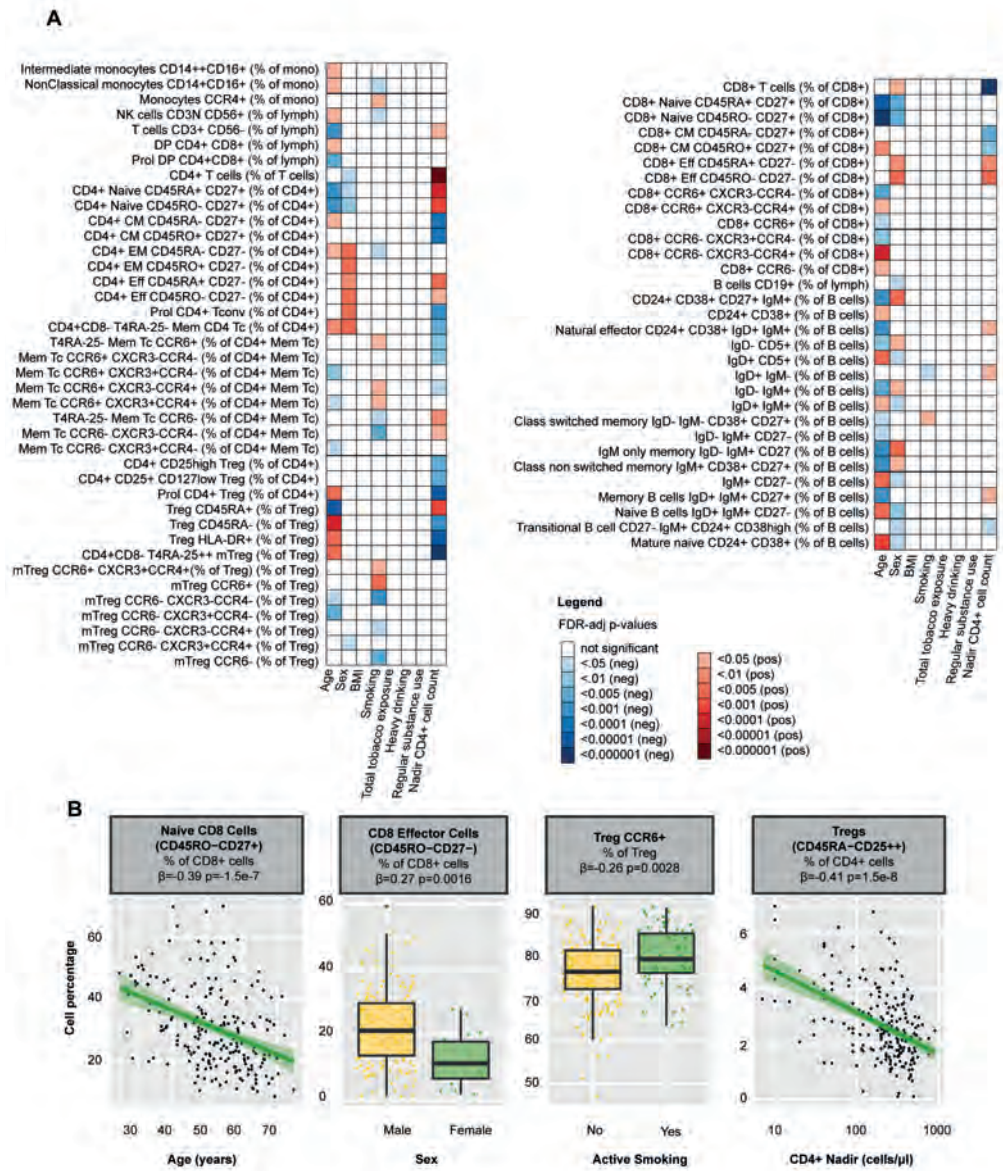


Figure 3. The HIV-1 reservoir, CMV serostatus and WBC percentages in PLHIV. (**A**) Heatmap of significant associations between WBC percentages (total n=108), CMV co-infection (serostatus and IgG titers in 198 seropositive PLHIV) and markers of the HIV-1 reservoir (CA -DNA and CA- RNA) in 211 PLHIV; WBC subsets that showed no significant correlations with any of the parameters (n=41) have been removed from the figure. (**B**) Examples of WBC percentages that were significantly associated with the viral reservoir or CMV IgG titers. Inverse-rank transformed data were analyzed using linear regression and adjusted for age, sex, sampling time, and season. For color coding of the FDR-adjusted p-values see legend. CA-DNA: CD4-cell-associated HIV-1 DNA; CA-RNA: CD4-cell-associated HIV-1 RNA; CMV: cytomegalovirus; PLHIV: people living with HIV; WBC: white blood cells.

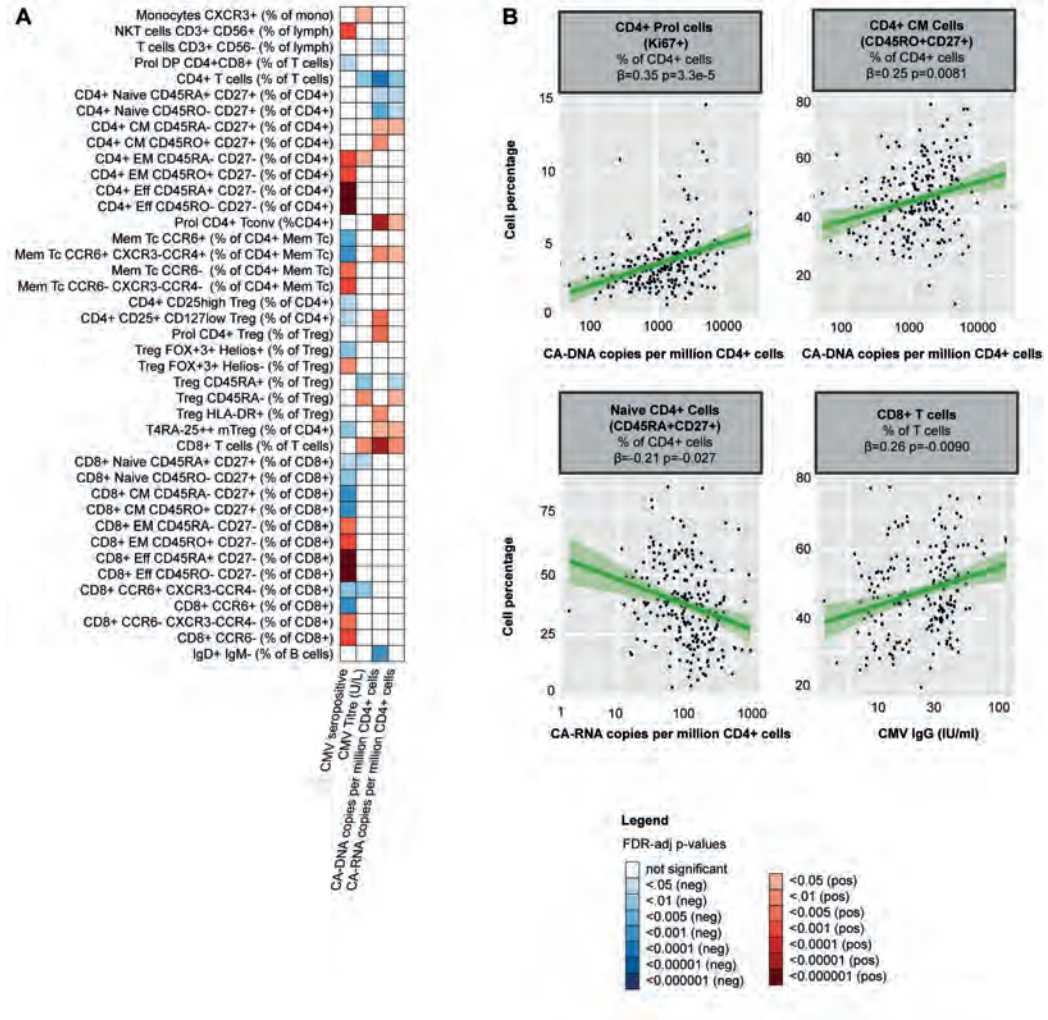


Figure 4. Inflammation, microbial translocation, and Th17 cells in PLHIV. (**A**) Boxplots showing differences in hsCRP and sCD14 between PLHIV (n=211) and healthy controls (n=56). (**B**) Heatmap showing associations between Th17 percentages, Th17/Treg ratios and sCD14, hsCRP, IFABP, and CCL20. (**C**) Associations between Th17 percentages and sCD14 and circulating IL-6 in PLHIV. (**D**) Levels of the microbial translocation marker IFABP in PLHIV and healthy controls. (**E**) Associations between IFABP and CA-RNA and CA-DNA in PLHIV. (**E**) Associations between CCL20 and circulating IL-17A and IFABP. Inverse-rank transformed data were analyzed using linear regression analyses and corrected for sampling time. CA-DNA: CD4-cell-associated HIV-1 DNA; CA-RNA: CD4-cell-associated HIV-1 RNA; CCL20: Chemokine (C-C motif) ligand 20; HC: healthy control; hsCRP: high-sensitivity CRP; IFABP: intestinal fatty-acid binding protein; IL-6: interleukin 6; IL-17A: interleukin 17A; Mem Tc: CD4+ memory T cell; PLHIV: people living with HIV; sCD14: soluble CD14; Th17 percentages: T-helper 17 cell percentages (Mem Tc CCR6+ CXCR3-CCR4+ as percentage of CD4+ Mem Tc); Treg: regulatory T cells; WBC: white blood cells.

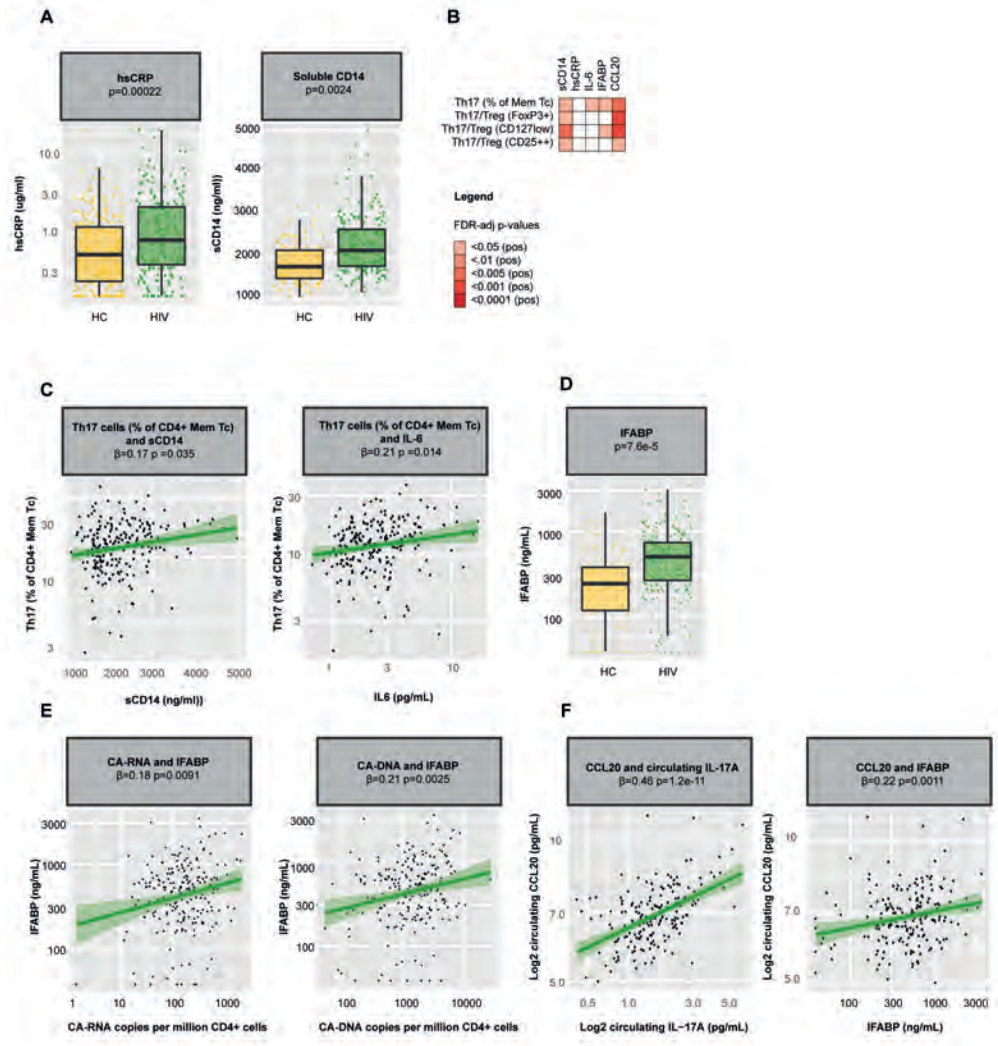


Figure 5. Functional consequences of WBC alterations in PLHIV. (**A**) Heatmap of NK(T) and T cell percentages (total n=71) that were significantly associated with the ex vivo production of IFN-γ, IL-17, and IL-22 upon ex vivo stimulation of PBMCs with seven different stimuli in 211 PLHIV; WBC subsets that showed no significant correlations with any of the parameters (n=44) have been removed from the figure. (**B**) Boxplot showing differences in ex vivo production capacity of IFN-γ upon seven days stimulation between PLHIV (n=211) and healthy controls (n=56). (**C**) Association between CA-DNA and ex vivo IFN-γ production capacity in PLHIV.(**D**) Association between CA-DNA and circulating IFN-γ in PLHIV. (***E***) Association between Th17 percentages and circulating IL-17A in PLHIV. (**F**) Heatmap of B cell percentages (total n=28) that were significantly correlated with IgM or IgG levels in PLHIV; WBC subsets that showed no significant correlations with any of the parameters (n=21) have been removed from the figure. Inverse-rank transformed data were analyzed using linear regression analyses and corrected for sampling time. For cohort comparisons, data were corrected for age, sex, sampling time, season and the proportion of CD4+ and CD8+ cells. For color coding of the FDR-adjusted p-values see legend. CA-DNA: CD4-cell-associated HIV-1 DNA; HC: healthy control; IFN-γ: interferon gamma; Ig: immunoglobulin; IL-17(A): interleukin 17(A); IL-22: interleukin 22; Mem Tc: CD4+ memory T cell; NK(T) cells: natural killer (T) cells; PBMC: peripheral blood mononuclear cell; PLHIV: people living with HIV; sCD14: soluble CD14; Th17 percentages: T-helper 17 cell percentages (Mem Tc CCR6+ CXCR3-CCR4+ as percentage of CD4+ Mem Tc); WBC: white blood cell

Second, we measured serum immunoglobulins and found significant correlations between serum IgM and IgM+ B cell populations (Figure 5F), but no cohort differences in IgM levels (10.04 [3.27] g/L in PLHIV versus 9.10 [3.79] g/L in controls, p= 0.12) or IgG levels (median [IQR] 0.76 [0.49] g/L in PLHIV versus 0.82 [0.56] g/L in controls, p= 0.22). These results indicate that while some of the alterations in adaptive immune function may be reversed by long-term cART[42,43], others, such as impaired IFN-γ responses, remain.

Discussion

In this study we show that, despite suppressive cART, the circulating innate and adaptive immune cell composition in PLHIV differs from that of HIV-uninfected individuals. We confirm that PLHIV exhibit a WBC profile characterized by proliferating memory and effector CD4+ and CD8+ T cells. While untreated HIV infection has been associated with a loss of circulating Th17 cells[10], we observed an expansion of circulating Th17 cells and increased Th17/Treg ratios during stable suppressive treatment, which was associated with plasma concentrations of IL-6, CCL20 and the microbial translocation marker IFABP. Furthermore, PLHIV showed clear changes in B cell maturation with reduced proportions of memory B cells and increased frequencies of plasmablasts. In the innate compartment, we observed an expansion of monocytes together with a loss of NK cells, specifically NK bright cells. In addition to age, sex, smoking, and CMV, we found strong associations between WBC populations and markers of the HIV-1 reservoir (CA-DNA and CA-RNA). Functionally, Th17 responses seemed to be preserved, whereas IFN-γ responses were compromised, especially in those with higher CA-DNA, which may affect host defense against some important bacterial pathogens (including *M. Tuberculosis*) as well as the HIV-1 reservoir.

Prior studies have shown that untreated HIV infection results in a massive depletion of Th17 cells from the peripheral blood and the mucosa[10], a process that may partially be reversed by cART[44,45]. We recently showed in the same cohort that PLHIV have a pro-inflammatory profile with increased monocyte-derived cytokines, particularly IL-1β[18]. IL-1β and IL-6 are among the critical cytokines driving differentiation of Th17[40], and we postulate that these cytokines may have contributed to the higher circulating Th17 numbers. Th17 cells in the peripheral blood poorly reflect mucosal Th17 numbers [46] and we cannot exclude that mucosal Th17 depletion with increased microbial translocation and altered Th17 recruitment occurs in the participants of our study. Concurrently to increased Th17 cells and pro-inflammatory Th17-like (CCR6+) mTregs, PLHIV showed increased peripheral blood Th17/Treg ratios. Increased Th17/Treg ratios have been linked to cardiovascular disease and atherosclerosis[31], cancer[32], and major depressive disorder[47], which are all highly prevalent in long-term treated PLHIV. Th17-mediated auto-immune diseases like psoriasis are also common among PLHIV, although they mostly occur during periods of severe immunosuppression and resolve upon

cART initiation[48]. Despite these changes in Th17, IL-17 and IL-22 cytokine responses did not differ between PLHIV and healthy controls. In contrast, we observed reduced *ex vivo* IFN-γ responses in PLHIV. IFN-γ is predominantly produced by NK(T) cells, Th1, and CD8+ cells[42]. Given that CD8+ cells were increased in PLHIV and Th1 cells did not differ between PLHIV and controls, we postulate that the reduced IFN-γ responses may have resulted from the loss of NK cells, which has been reported previously in both untreated and treated PLHIV[49,50]. In line with prior data, we observed an inverse relationship between IFN-γ responses and CA-DNA, suggesting that the failure to restore the NK cell compartment after cART initiation may be important for the containment of the HIV-1 reservoir[51].Moreover, IFN-γ is a key cytokine in the immune response against *M. tuberculosis* which remains an important pathogen in treated PLHIV .[52]. Improving NK-derived IFN-γ responses, may therefore be relevant in the context of *M. Tuberculosis* and HIV cure.

Different factors may contribute to the variation in T-cells repertoire in PLHIV. First, the effects of demographic factors such as age, sex, and smoking resembled those observed previously in healthy individuals[17,39]. Second, PLHIV in our study were almost universally coinfected with CMV, and CMV seropositivity was associated with the expansion of effector and EM T cells [39,53]. Of note, high CMV IgG levels may reflect frequent CMV reactivations or result from a stronger immune response (including adequate B/T cell interactions and B cell responses) and, consequently, fewer activations[54]. Importantly, higher CMV IgG titers have been linked to the development of non-AIDS-defining events such as cardiovascular disease[55]. Third, we found substantial associations WBC subsets and CD4+ nadir and markers of the HIV-1 reservoir. We found detectable levels of CA-RNA and CA-DNA in almost all participants. Overall, CA-DNA showed more and stronger correlations with WBC subsets than did CA-RNA, which can be explained by the fact that CA-RNA reflects transcriptional activity, which is more variable than CA-DNA, which equals integrated DNA[11,56].

Next to T cell dysfunction, HIV is characterized by aberrant B cell responses and B cell dysfunction. Using a different set of B cell markers than earlier studies in PLHIV [57,58], we confirm their observations that proportions of naïve B cells are increased and memory B cells are reduced in PLHIV. Moreover, adequate B cell maturation requires optimal communication between T and B cells, which, according to our data, might be compromised in chronic stable PLHIV. B/ T cell interactions take place in the germinal centers in lymph nodes and are orchestrated by follicular Th cells (Tfh)[59]. As these cells are known to be highly permissive to HIV infection and serve as reservoirs during chronic infection, they could potentially explain these disrupted B/T cell interactions[59,60]. Clinically, compromised B/T cells interactions may contribute to impaired immune responses to vaccination as well as increased risks for infections, such as invasive pneumococcal disease[61].

Several limitations should be considered when interpreting our data. First, the observational study design limits causal inferences. Second, generalizability of our findings to women, children and non-European populations requires further studying. Third, individuals in our control group were younger and more often female. However, we controlled for these cohort differences in our statistical models. Fourth, we used a predefined set of flow cytometry panels. While this standardized approach enhances validity and reproducibility and enabled us to compare our results with those of a large cohort of healthy controls, some interesting WBC subsets and markers for HIV have not been included (e.g. Tfh cells and PD-1/CD57 expression in the context of B/T cell interactions and immune cell exhaustion respectively). Finally, our WBC data are limited to the peripheral blood and we have no data on tissue-specific WBC composition (e.g. lymph nodes or the gut).

In summary, we show that the circulating innate and adaptive immune cell composition is altered in a large group of long-term treated PLHIV. Furthermore, our findings suggest that some of the adaptive immune responses (Th17) are preserved while IFN-γ responses are compromised. Our comprehensive approach provides new insight into the changes in the immune cell architecture and functional immunity in long-term treated HIV and highlights associations with the HIV-reservoir, underlining the need for early cART initiation. Our results are currently validated and extended in a multi-omics study including 2000 virally suppressed PLHIV (clinicaltrials.gov identifier: NCT03994835).

Supplementary data

All supplementary figures, the supplementary methods, and supplementary table 1-3 can be found at the end of this manuscript. Supplementary tables 4-10 are digitally available at the website of the published article..

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Table 1. General characteristics

Characteristic	PLHIV (n = 211)	HC (n = 56)	P-value
Age, years	52.5 (13.2)	30.0 (27.1)	<0.0001
Sex, female, n/N (%)	19/211 (9.0)	22/56 (39.3)	<0.0001
BMI, kg/m²	24.1 (4.0)	23.6 (3.6)	0.41
Time since HIV diagnosis, years	8.5 (9.5)	-	-
Way of transmission, n/N (%)		-	-
MSM	159/211 (75.4)	-	-
Heterosexual contact	9 (4.3)	-	-
IDU	3 (1.4)	-	-
Other or unknown	40 (19.0)		
Nadir CD4⁺ cell count, cells/µl	250 (230)	-	-
CD4⁺ count, cells/µl	660 (330)	-	-
Zenith HIV-RNA, copies/mL	100 000 (341 182)	-	-
HIV-RNA >50 copies/mL ≤1 yr. prior to inclusion, n/N (%)	23/210 (11.0)	-	-
CA-DNA (copies per million CD4+ cells)	1547 (2270)		
CA-RNA (copies per million CD4+ cells)	157 (224)		
cART-naive, n/N (%)	30/211 (14.2)	-	-
Time on cART, years	6.6 (7.7)	-	-
ARV classes, n/N (%)			
NNRTI	63/211 (29.9)	-	-
PI	32/211 (15.2)	-	-
INSTI	141/211 (66.8)	-	-
Co-medication, n/N (%)			
Cholesterol lowering drugs	58/211 (27.5)	-	-
Antihypertensive drugs	50/211 (23.7)	-	-
Metformin	9/211 (4.3)	-	-
Active smoking, n/N (%)	63/211 (29.9)	2/56 (3.6)	<0.0001
Heavy drinking, n/N (%)*	28/211 (13.3)	11/56 (19.6)	0.29
Regular substance use, n/N (%)†	61/211 (28.9)	3/56 (5.4)	<0.0001

Data depicted as median (IQR) unless stated otherwise. Data analyzed using Mann-Whitney U or χ^2 (or Fisher's exact) where applicable.

*Classified according to the CDC definition: for men, ≥15 drinks per week and for women, ≥8 drinks/week.

†Defined as use of any psychoactive substance (with the exception of alcohol and tobacco) during periods ≥ 1 time per week including ≥ 1 time during the 30 days prior to the study visit.

ARV, antiretroviral drug; BMI, body mass index CA-DNA: CD4-cell-associated HIV-1 DNA; CA-RNA: CD4-cell-associated HIV-1 RNA; HC: healthy control; cART, combination antiretroviral therapy; INSTI, integrase inhibitor; IDU, intravenous drug use; MSM, men who have sex with men; NNRTI, non-nucleoside reverse transcriptase inhibitor; PLHIV: people living with HIV; PI, protease inhibitor

Table 2a. Median (IQR) WBC percentages and WBC numbers in HIV and healthy controls – Innate cells and CD8+ T cells

Cell population		Percentages			WBC numbers/mL		
		PLHIV (n=211)	HC (n=56)	FDR-adj	PLHIV (n=211)	HC (n=56)	FDR-adj
	% of	Median (IQR)	Median (IQR)	P-value	Median (IQR)	Median (IQR)	P-value
Leukocytes CD45+	NA	NA	NA	NA	6500000 (2850000)	6100000 (2300000)	0.032
Neutrophils	leukocytes	60.1 (17.6)	59.3 (16.2)	0.92	3795757 (2345350)	3536984 (1980831)	0.19
Monocytes CD14+	leukocytes	8 (2.5)	7.6 (2.5)	0.024	504887 (299749)	463562 (236696)	0.0017
Classical monocytes CD14++CD16-	monocytes	66.8 (12.1)	66.5 (14.3)	0.42	347013 (239266)	313568 (195346)	0.0027
Intermediate monocytes CD14++CD16+	monocytes	9.1 (7.6)	8.2 (7.3)	0.50	44708 (40244)	39405 (37311)	0.14
NonClassical monocytes CD14+CD16+	monocytes	6.6 (3.6)	7.3 (3.6)	$-5.8\cdot10^{-7}$	31459 (20806)	31238 (20794)	-0.14
Monocytes CXCR3+	monocytes	4.8 (3.4)	4.3 (3.1)	0.010	20542 (16860)	15285 (10906)	$4.7\cdot10^{-10}$
Monocytes CCR4+	monocytes	81 (10.5)	75.8 (14.3)	$3.0\cdot10^{-8}$	352612 (289891)	253758 (202853)	$3.6\cdot10^{-8}$
Monocytes CCR6+	monocytes	4.5 (3.4)	5.4 (4.8)	-0.014	19689 (15640)	18499 (14406)	0.30
Lymphocytes	leukocytes	29.6 (15.2)	30.9 (15.2)	-0.90	1858207 (1068309)	1837099 (1068810)	-0.14
NK cells CD3- CD56+	lymphocytes	10.2 (8.1)	10.8 (7.8)	$-3.5\cdot10^{-6}$	176372 (163007)	204295 (194495)	-0.0044
NK dim CD56+ CD16+	NK cells	94.9 (4.4)	93.5 (6.1)	0.12	166680 (163357)	191415 (193283)	-0.0084
NK bright CD56++ CD16-	NK cells	2.7 (3)	4.2 (3.9)	-0.030	5387 (4458)	8246 (7422)	$-4.4\cdot10^{-8}$
NK CD56+ CD16-	NK cells	2 (2.2)	2.2 (2.2)	-0.48	3459 (2558)	5097 (4152)	$-6.2\cdot10^{-5}$
NKT cells CD3+ CD56+	lymphocytes	4.7 (4.8)	3.8 (4.9)	0.0076	83299 (100330)	69482 (106511)	0.00036
T cells CD3+ CD56-	lymphocytes	72.7 (10.8)	73.3 (9.8)	0.0022	1359057 (833573)	1335918 (865890)	-0.028
DP CD4+ CD8+	T cells	0.9 (0.6)	0.8 (0.8)	0.72	12723 (10937)	12350 (10912)	0.19
Prol DP CD4+CD8+	T cells	7.9 (4.6)	7 (6)	0.00021	859 (958)	642 (861)	0.0067
DN CD4-CD8-	T cells	3 (2.2)	2.9 (2.3)	-0.54	36399 (42821)	37022 (36843)	-0.051
Prol DN CD4-CD8-	T cells	11.6 (7.6)	9.7 (5.5)	0.017	65321 (42051)	52655 (39738)	0.11
CD8+ T cells	T cells	46.3 (16.4)	28.1 (12.1)	$2.7\cdot10^{-48}$	604943 (442205)	370612 (245431)	$1.4\cdot10^{-26}$
CD8+ Naive CD45RA+ CD27+	CD8+ cells	27.5 (22.8)	59.7 (26.9)	$-5.9\cdot10^{-23}$	169941 (136534)	197620 (210634)	-0.0095
CD8+ Naive CD45RO- CD27+	CD8+ cells	29.1 (19.4)	56.3 (27.9)	$-7.5\cdot10^{-15}$	161961 (144626)	183621 (208158)	$-1.1\cdot10^{-5}$
CD8+ CM CD45RA- CD27+	CD8+ cells	33 (15.6)	25.6 (18.8)	0.72	180206 (141961)	88720 (57399)	$2.8\cdot10^{-24}$
CD8+ CM CD45RO+ CD27+	CD8+ cells	33.3 (15)	31.2 (18)	0.022	189251 (136407)	105590 (59429)	$2.1\cdot10^{-22}$
CD8+ EM CD45RA- CD27-	CD8+ cells	12.8 (12.3)	3.7 (3.8)	$1.2\cdot10^{-15}$	64656 (81375)	11479 (16098)	$2.0\cdot10^{-27}$
CD8+ EM CD45RO+ CD27-	CD8+ cells	11.6 (9.3)	4.2 (4.5)	$1.0\cdot10^{-13}$	62749 (75399)	13073 (20176)	$5.9\cdot10^{-29}$

CD8+ Eff CD45RA+ CD27-	CD8+ cells	19.2 (15.2)	4.1 (8.7)	$9.3 \cdot 10^{-20}$	105635 (126272)	16171 (26713)	$5.4 \cdot 10^{-34}$
CD8+ Eff CD45RO- CD27-	CD8+ cells	20.6 (16)	3.3 (8.1)	$2.8 \cdot 10^{-26}$	116350 (130051)	12720 (25448)	$7.0 \cdot 10^{-39}$
Prol CD8+	CD8+ cells	4.2 (2.8)	2.9 (1.9)	0.00089	28593 (32389)	15812 (16330)	$4.0 \cdot 10^{-14}$
CD8+ CCR6+ CXCR3-CCR4-	CD8+ cells	2.6 (2.9)	7.4 (6.3)	$-6.2 \cdot 10^{-24}$	28466 (31569)	45048 (45366)	-0.013
CD8+ CCR6+ CXCR3+CCR4-	CD8+ cells	0.9 (1)	0.6 (0.7)	$6.6 \cdot 10^{-7}$	9784 (12912)	4093 (6592)	$2.6 \cdot 10^{-23}$
CD8+ CCR6+ CXCR3-CCR4+	CD8+ cells	0.6 (0.5)	2 (2)	$-2.3 \cdot 10^{-40}$	6579 (6561)	11886 (12668)	$-2.8 \cdot 10^{-9}$
CD8+ CCR6+ CXCR3+CCR4+	CD8+ cells	0.2 (0.3)	0.3 (0.5)	-0.007	1721 (2763)	1772 (3627)	0.061
CD8+ CCR6+	CD8+ cells	4.6 (4.1)	11.2 (8.2)	$-2.8 \cdot 10^{-27}$	49172 (49068)	68559 (54330)	-0.18
CD8+ CCR6- CXCR3-CCR4-	CD8+ cells	75.1 (13.8)	65.5 (17.3)	$1.0 \cdot 10^{-12}$	770332 (574681)	400725 (297371)	$2.8 \cdot 10^{-36}$
CD8+ CCR6- CXCR3+CCR4-	CD8+ cells	9.6 (9.7)	4.2 (4.8)	$2.1 \cdot 10^{-32}$	111832 (132371)	24734 (37593)	$5.1 \cdot 10^{-46}$
CD8+ CCR6- CXCR3-CCR4+	CD8+ cells	7.4 (6.5)	15.8 (15.4)	$-2.5 \cdot 10^{-22}$	76775 (83200)	86969 (116090)	-0.25
CD8+ CCR6- CXCR3+CCR4+	CD8+ cells	0.9 (1.1)	1 (1.9)	-0.72	8615 (12660)	5770 (13629)	$9.6 \cdot 10^{-6}$
CD8+ CCR6-	CD8+ cells	95.4 (4.1)	88.8 (8.2)	$3.3 \cdot 10^{-27}$	989581 (704211)	524146 (379660)	$1.0 \cdot 10^{-35}$

Table 2b. Median (IQR) WBC percentages and WBC numbers in HIV and healthy controls – CD4+ T cells

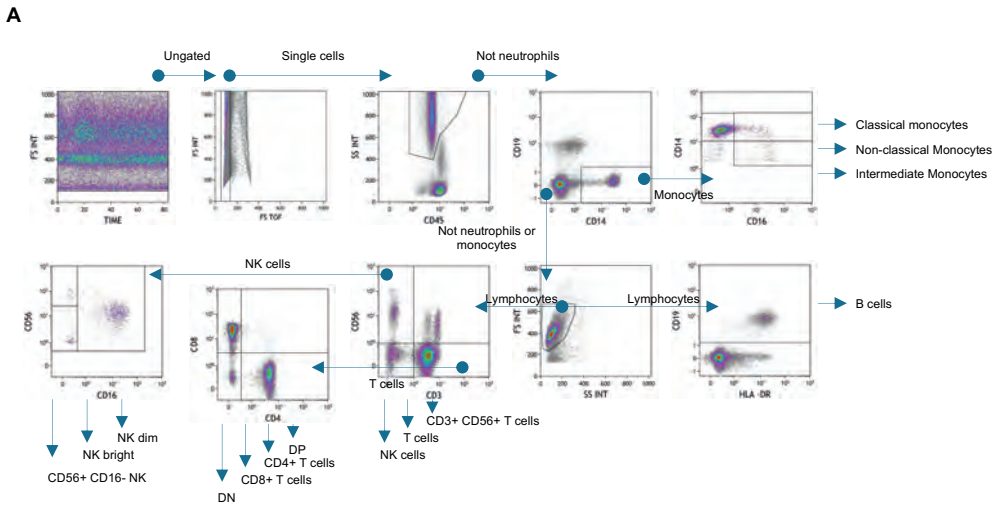
		Percentages			WBC numbers/mL		
		PLHIV (n=211)	HC (n=56)	FDR-adj	PLHIV (n=211)	HC (n=56)	FDR-adj
Cell population	**% of**	**Median (IQR)**	**Median (IQR)**	**P-value**	**Median (IQR)**	**Median (IQR)**	**P-value**
CD4+ T cells	T cells	49.7 (17.7)	66.7 (13.8)	$-1.9 \cdot 10^{-42}$	659219 (442798)	886881 (655896)	$-8.9 \cdot 10^{-5}$
CD4+ Naive CD45RA+ CD27+	CD4+ cells	37.2 (24.8)	51.3 (18.2)	-0.0001	209761 (251211)	406257 (451223)	$-3.7 \cdot 10^{-6}$
CD4+ Naive CD45RO- CD27+	CD4+ cells	37.6 (21.9)	52.1 (16.6)	-0.0007	212335 (238439)	404432 (418855)	$-1.9 \cdot 10^{-5}$
CD4+ CM CD45RA- CD27+	CD4+ cells	46.5 (19.5)	43.2 (15.6)	0.81	253493 (161091)	336462 (216832)	-0.00030
CD4+ CM CD45RO+ CD27+	CD4+ cells	45.3 (16.7)	42.7 (14.1)	0.32	247219 (158618)	347104 (228325)	$-2.3 \cdot 10^{-5}$
CD4+ EM CD45RA- CD27-	CD4+ cells	9.1 (9.8)	4.4 (3.1)	$1.9 \cdot 10^{-9}$	52042 (52950)	34935 (25271)	0.00036
CD4+ EM CD45RO+ CD27-	CD4+ cells	9.1 (10.2)	4.5 (3.1)	$8.9 \cdot 10^{-12}$	51500 (53043)	35632 (23215)	$3.3 \cdot 10^{-5}$
CD4+ Eff CD45RA+ CD27-	CD4+ cells	1.8 (3.5)	0.1 (0.4)	$2.6 \cdot 10^{-20}$	9817 (22947)	983 (3095)	$3.5 \cdot 10^{-16}$
CD4+ Eff CD45RO- CD27-	CD4+ cells	1.7 (3.4)	0.2 (0.3)	$8.8 \cdot 10^{-25}$	10036 (19881)	1481 (2326)	$6.6 \cdot 10^{-19}$
Prol CD4+ Tconv	CD4+ cells	3.4 (1.9)	2.2 (1.2)	$7.4 \cdot 10^{-9}$	20608 (11139)	18773 (14585)	0.019
CD4+CD8- Mem CD4 Tc	CD4+ cells	26.2 (11.9)	13.3 (7.8)	$8.8 \cdot 10^{-25}$	275908 (155905)	192481 (157213)	$2.7 \cdot 10^{-10}$
Mem Tc CCR6+	CD4+ Mem Tc	37.3 (13.8)	39.7 (15.4)	-0.90	100004 (64072)	68230 (62727)	$2.9 \cdot 10^{-9}$
Mem Tc CCR6+ CXCR3-CCR4-	CD4+ Mem Tc	13.2 (8.1)	14.1 (9.3)	-0.0037	33549 (30517)	25313 (26730)	0.0023
Mem Tc CCR6+ CXCR3+CCR4-	CD4+ Mem Tc	5.9 (6.4)	7.6 (6.8)	-0.49	17350 (19505)	14070 (18071)	0.00099
Mem Tc CCR6+ CXCR3-CCR4+	CD4+ Mem Tc	12.7 (7.4)	10.9 (6.2)	0.00013	34123 (25400)	18387 (19825)	$3.4 \cdot 10^{-14}$
Mem Tc CCR6+ CXCR3+CCR4+	CD4+ Mem Tc	3.3 (3.7)	3.4 (4)	-0.16	8872 (11475)	6783 (8170)	$3.6 \cdot 10^{-8}$
Mem Tc CCR6-	CD4+ Mem Tc	62.7 (13.8)	60.3 (15.4)	0.90	167774 (123186)	115211 (98546)	$2.9 \cdot 10^{-9}$
Mem Tc CCR6- CXCR3-CCR4-	CD4+ Mem Tc	35.9 (20.1)	28.6 (16.3)	0.077	101943 (84588)	58901 (56747)	$1.2 \cdot 10^{-8}$
Mem Tc CCR6- CXCR3+CCR4-	CD4+ Mem Tc	7.2 (7)	9.1 (9.2)	-0.82	19165 (24275)	17319 (22995)	0.00012
Mem Tc CCR6- CXCR3-CCR4+	CD4+ Mem Tc	12.2 (6.9)	14.7 (8.6)	-0.0002	33972 (29400)	27813 (23793)	0.00028
Mem Tc CCR6- CXCR3+CCR4+	CD4+ Mem Tc	2.4 (3.2)	2.8 (4.2)	-0.90	6733 (10531)	5774 (8843)	$7.8 \cdot 10^{-5}$
CD4+ CD25high Treg	CD4+ cells	4.6 (2.2)	3.7 (1.6)	0.0035	26988 (19945)	34890 (26594)	-0.055
CD4+ CD25+ CD127low Treg	CD4+ cells	6.6 (2.8)	6.5 (2)	0.49	35991 (23239)	50680 (32936)	-0.0014

Prol CD4+ Treg	Treg	16.8 (7.9)	14.7 (7.3)	0.077	6596 (3914)	8880 (6010)	$-4.0\cdot10^{-8}$
Treg FOX+3+ Helios+	Treg	75.7 (10.5)	65.9 (11.2)	$8.4\cdot10^{-19}$	29289 (22601)	37759 (29121)	-0.072
Treg FOX+3+ Helios-	Treg	8.7 (3.8)	15.7 (6.6)	$-2.5\cdot10^{-38}$	3519 (3222)	9370 (7377)	$-5.4\cdot10^{-28}$
Treg CD45RA+	Treg	24 (17.5)	33.6 (22.5)	-0.38	9758 (11206)	18291 (24802)	-0.0031
Treg CD45RA-	Treg	76 (17.5)	66.4 (22.5)	0.38	29309 (16817)	37471 (26330)	-0.00021
Treg HLA-DR+	Treg	42.7 (15.6)	33.1 (16.3)	0.053	16561 (9658)	20465 (13710)	-0.0022
CD4+CD8- T4RA-25++ mTreg	CD4+ cells	2.5 (1.7)	1.7 (1)	0.0016	27163 (17779)	25060 (15980)	0.72
mTreg CCR6+ CXCR3-CCR4-	Treg	3.5 (3.4)	3.9 (4.2)	-0.00010	894 (1042)	968 (1035)	-0.0021
mTreg CCR6+ CXCR3+CCR4-	Treg	0.5 (0.9)	0.7 (1.2)	-0.00020	120 (260)	190 (360)	-0.00099
mTreg CCR6+ CXCR3-CCR4+	Treg	65.3 (11.8)	58.8 (14.4)	$1.4\cdot10^{-7}$	17318 (12846)	14172 (9332)	0.026
mTreg CCR6+ CXCR3+CCR4+	Treg	6.7 (7.6)	6.9 (8.5)	-0.37	1632 (2766)	1594 (2508)	-0.45
mTreg CCR6+	Treg	77.3 (9.3)	74.5 (12.7)	0.010	20227 (15071)	18375 (12700)	0.38
mTreg CCR6- CXCR3-CCR4-	Treg	3.9 (3.3)	6 (4.9)	$-1.6\cdot10^{-8}$	948 (1214)	1507 (1453)	$-3.7\cdot10^{-5}$
mTreg CCR6- CXCR3+CCR4-	Treg	0.5 (0.8)	0.7 (1.3)	-0.078	125 (256)	178 (375)	-0.13
mTreg CCR6- CXCR3-CCR4+	Treg	15.4 (7.7)	15.5 (9.9)	-0.90	3848 (3927)	3836 (3330)	0.81
mTreg CCR6- CXCR3+CCR4+	Treg	1.4 (2.1)	1.4 (2)	-0.20	418 (598)	366 (625)	-0.23
mTreg CCR6-	Treg	22.7 (9.3)	25.5 (12.7)	-0.010	5890 (4743)	6462 (5385)	-0.28

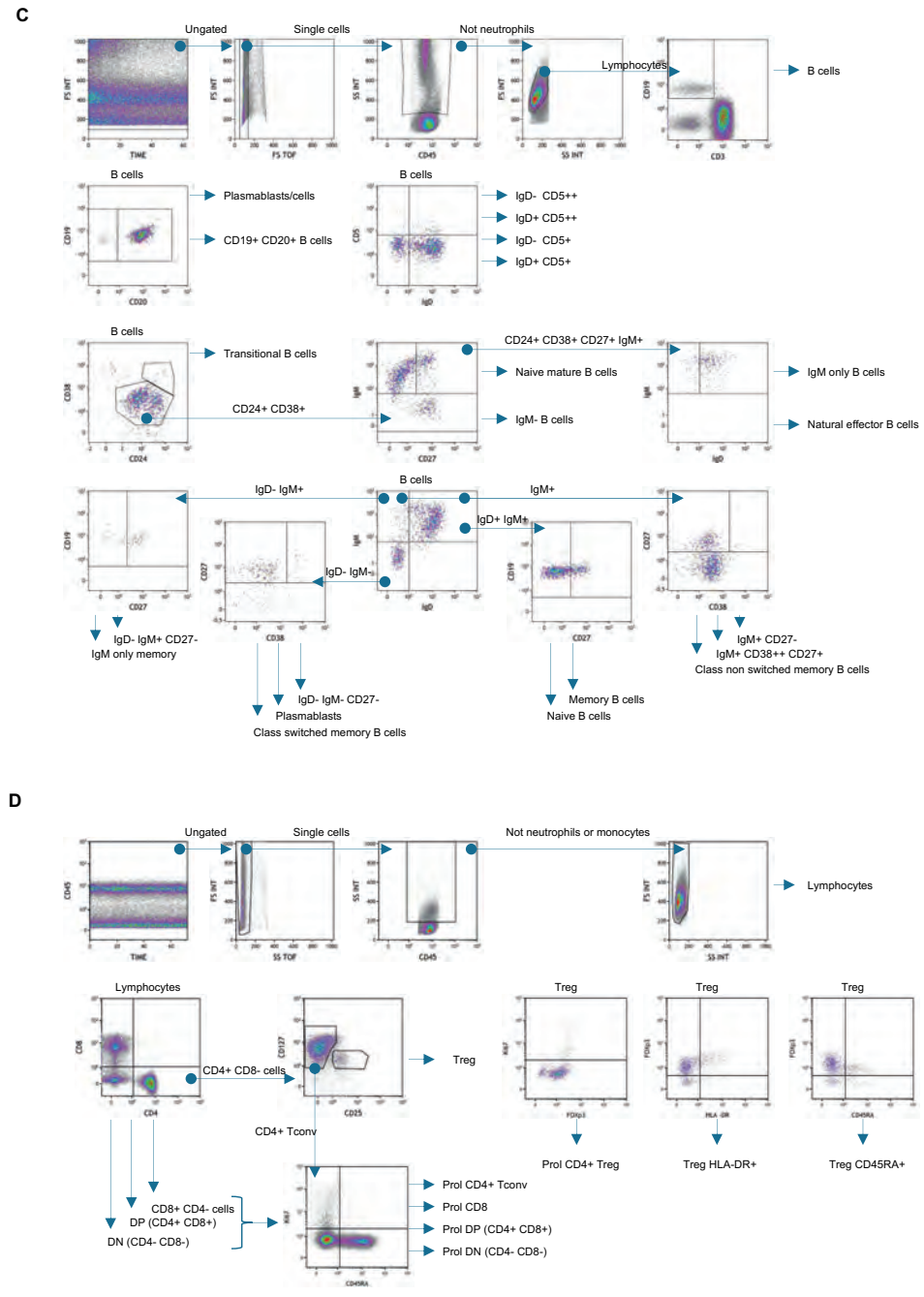
Table 2c. Median (IQR) WBC percentages and WBC numbers in HIV and healthy controls – B cells

Cell population		Percentages			WBC numbers/mL		
		PLHIV (n=211)	HC (n=56)	FDR-adj	PLHIV (n=211)	HC (n=56)	FDR-adj
	% of	Median (IQR)	Median (IQR)	P-value	Median (IQR)	Median (IQR)	P-value
B cells CD19+	lymphocytes	6.2 (4.1)	6.4 (3.3)	0.23	113016 (100381)	125472 (90421)	-0.062
CD19+ CD20- Plasma blasts/cells	B cells	1.8 (2.5)	1.6 (1.8)	0.23	1078 (1228)	941 (1198)	0.0013
CD19+ CD20+ B cells	B cells	98.2 (2.5)	98.4 (1.8)	-0.23	56125 (52048)	59534 (53989)	0.032
IgM only B cells	B cells	2.5 (2.9)	1.4 (1.6)	$1.0\cdot10^{-8}$	1427 (1780)	800 (1111)	$1.4\cdot10^{-11}$
CD24+ CD38+	B cells	84.8 (6.2)	85 (6.7)	0.12	50133 (46705)	50353 (45567)	0.020
Natural effector	B cells	8 (7.5)	11.7 (7.7)	$-1.8\cdot10^{-5}$	4864 (5444)	6953 (7617)	-0.30
IgD- CD5++	B cells	0.8 (0.8)	0.7 (0.6)	0.12	513 (503)	429 (440)	0.00048
IgD+ CD5++	B cells	3.8 (2.9)	6.3 (4.7)	$-4.8\cdot10^{-10}$	2268 (3151)	3614 (5046)	-0.011
IgD- CD5+	B cells	25.3 (18.3)	19.8 (10.6)	$5.1\cdot10^{-5}$	14388 (13968)	11838 (9937)	$7.7\cdot10^{-7}$
IgD+ CD5+	B cells	69.4 (16.5)	72.1 (11.4)	-0.12	40139 (40248)	43935 (40860)	-0.17
IgM-	B cells	16 (12.5)	15.2 (10.9)	0.15	9479 (10053)	8954 (9073)	0.0040
IgD- IgM-	B cells	19.4 (15.5)	16.3 (10.4)	0.027	11396 (11844)	9709 (8642)	0.00029
IgD+ IgM-	B cells	2.6 (4.3)	3.8 (5.5)	-0.010	1546 (2842)	2591 (3852)	-0.37
IgD- IgM+	B cells	5.4 (5.1)	3 (3)	$2.1\cdot10^{-12}$	3262 (3273)	1755 (2336)	$1.9\cdot10^{-14}$
IgD+ IgM+	B cells	69.7 (18)	73.2 (12.3)	-0.0027	40745 (38917)	44630 (43280)	-0.25
Class switched memory	B cells	13.6 (12.2)	11.4 (8.6)	0.082	7555 (9176)	7222 (6657)	0.0014
IgD- IgM- CD27-	B cells	3.5 (2.4)	2.7 (1.8)	0.017	2193 (2120)	1692 (1551)	0.00017
Plasmablast (IgD- IgM- CD38++)	B cells	1.2 (1.9)	1.1 (1.3)	0.33	701 (1053)	628 (797)	0.010
IgD- IgM+ CD27-	B cells	2 (1.9)	0.8 (0.9)	$4.6\cdot10^{-27}$	1117 (1230)	421 (764)	$1.2\cdot10^{-23}$
IgM only memory (IgD- IgM+ CD27)	B cells	3.5 (4.1)	2.2 (2.4)	$7.8\cdot10^{-6}$	1963 (2398)	1289 (1477)	$6.5\cdot10^{-9}$
Class non switched memory	B cells	12.7 (12)	15 (11.3)	0.077	7299 (7663)	8611 (10109)	-0.47
IgM+ CD38++ CD27+	B cells	0.5 (0.5)	0.6 (0.4)	0.93	319 (373)	315 (350)	0.12
IgM+	B cells	61.7 (24.1)	60.5 (18.4)	0.93	35665 (34879)	36868 (32233)	-0.093
Memory B cells (IgD+ IgM+ CD27+)	B cells	9.1 (8.1)	19.4 (10)	$-2.4\cdot10^{-29}$	5366 (6059)	11082 (10748)	$-9.7\cdot10^{-9}$
Naive B cells (IgD+ IgM+ CD27-)	B cells	58.9 (23.7)	53.6 (19.2)	0.0087	34361 (34682)	32723 (28980)	0.0078
Transitional B cells (CD24++ CD38++)	B cells	3.4 (3.6)	5.2 (3.4)	-0.0002	2171 (2455)	2949 (3350)	-0.31
IgM+ CD27-	B cells	5.8 (4.9)	6.9 (5.1)	-0.023	3494 (4638)	4097 (4400)	-0.94
Naive mature B cells	B cells	41 (21.7)	41.7 (16)	0.99	24476 (25762)	24472 (22758)	-0.15

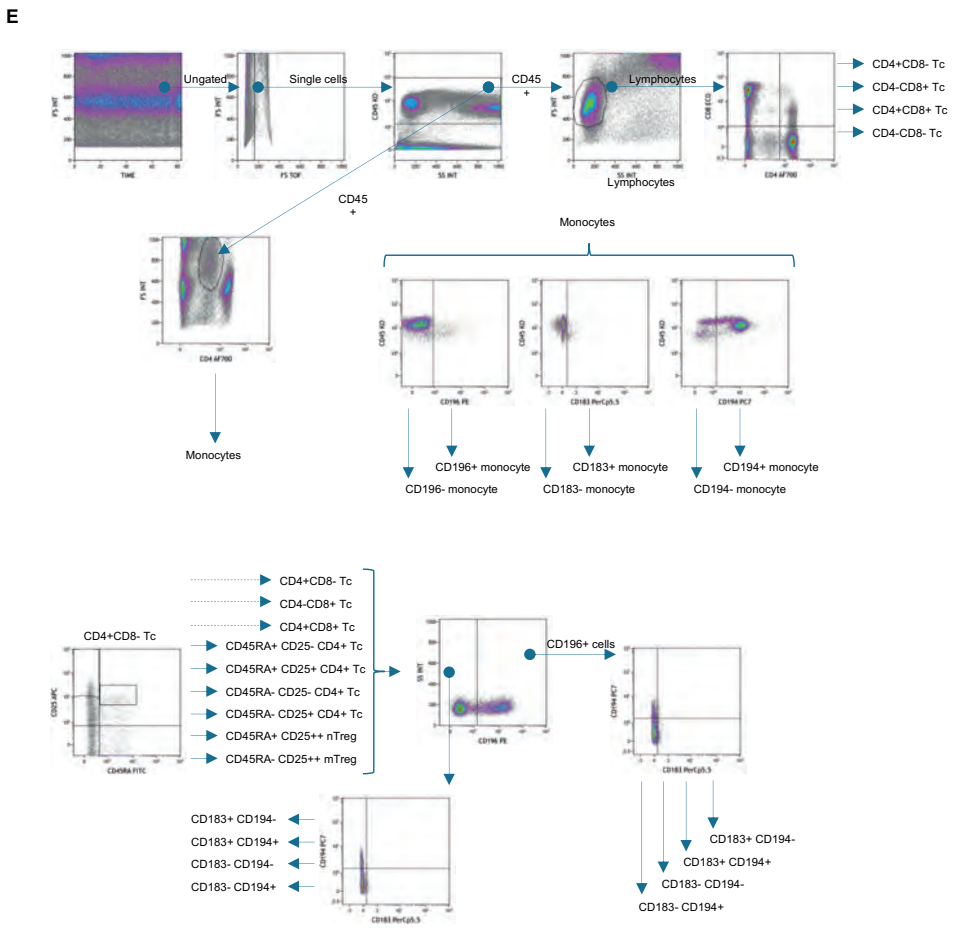
Inverse-rank transformed data were analyzed using linear regression and adjusted for age, sex, sampling time, and season.
HC: healthy control; PLHIV: people living with HIV; WBC: white blood cells



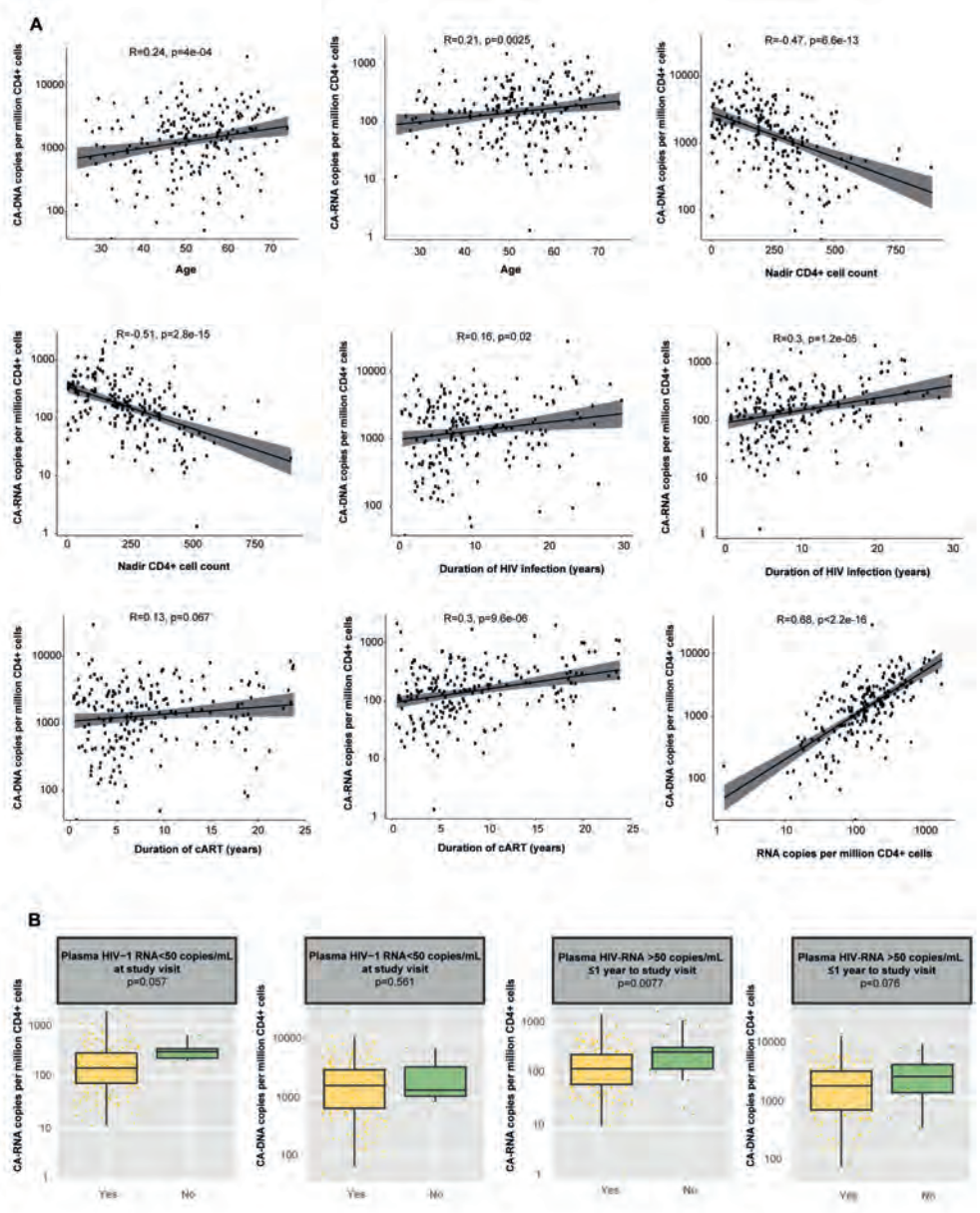
Supplementary Figure 1. Flow cytometry gating strategies of the general (A) and T cell (B) panels



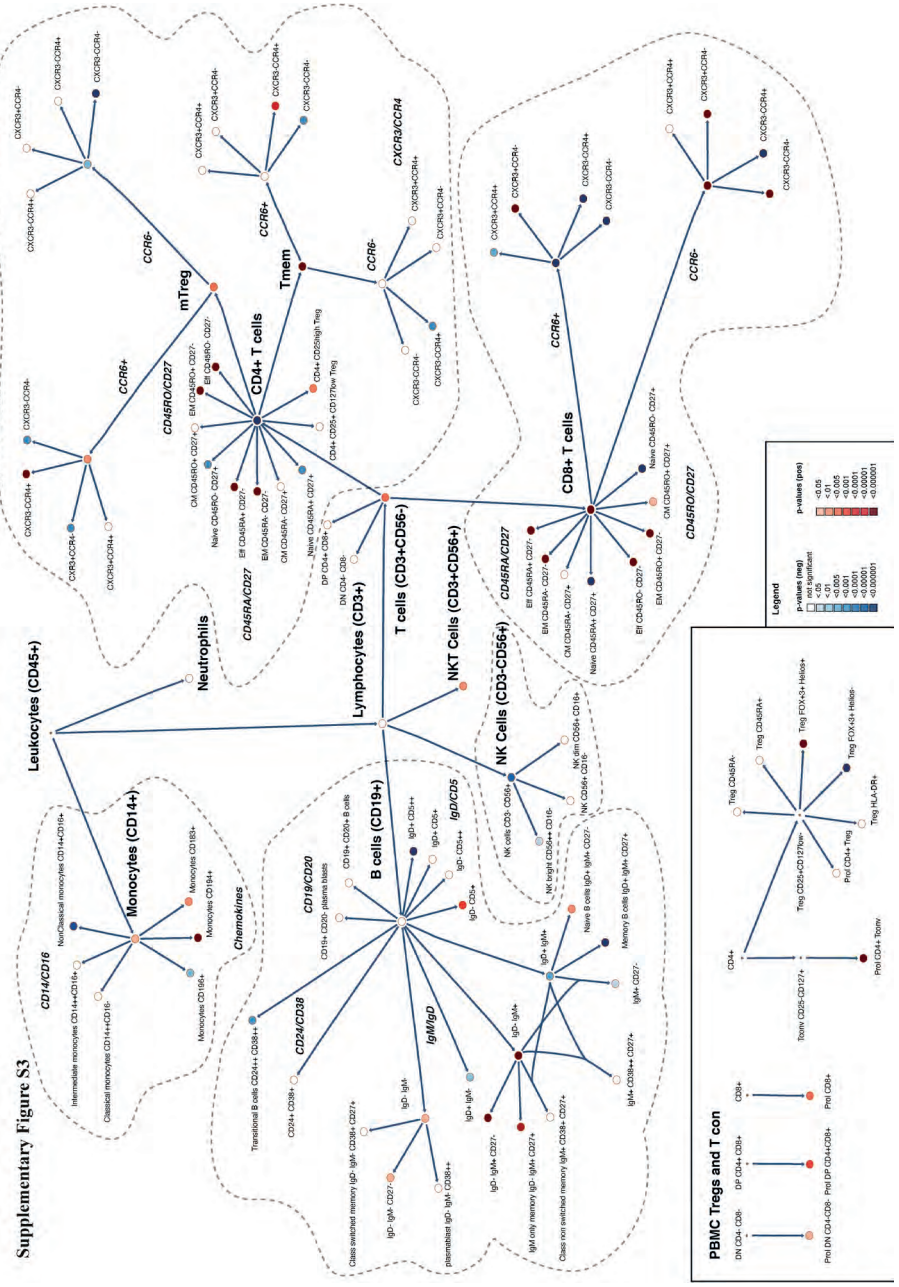
Supplementary Figure 1. Flow cytometry gating strategies of the B cell (C) and Treg panels



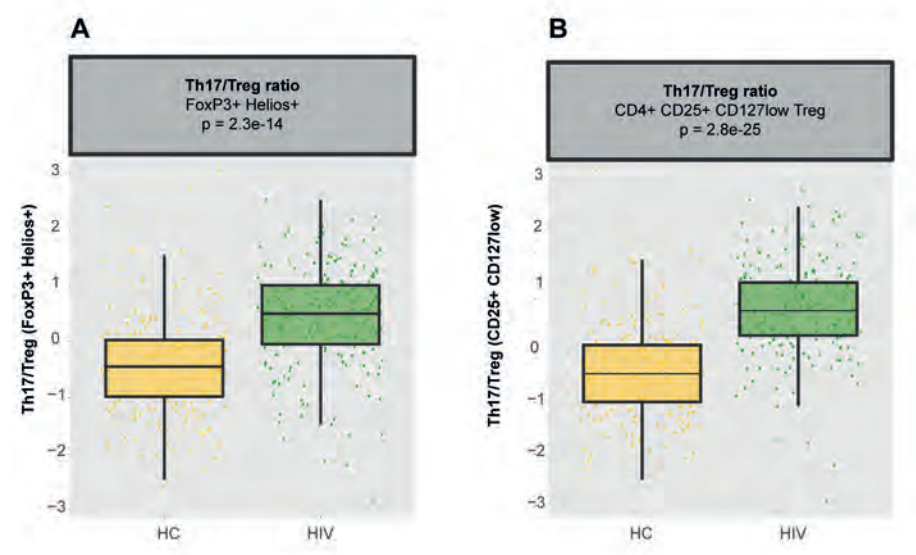
Supplementary Figure 1. Flow cytometry gating strategies of the chemokine receptor (CCR) panel (E)



Supplementary Figure 2. (**A**) Spearman's correlations between CA-DNA and CA-RNA and clinical factors (**B**) Differences in CA-RNA and CA-RNA between PLHIV with (205/210 [98%]) and without HIV-1 RNA 50 <copies/mL (5/210 [2%]) at study visit and between PLHIV with (23/210 [11%]) and without plasma HIV-RNA>50 copies/mL (187/210 [89%]) in the year prior to study visit.CD-DNA: CD4-cell associated HIV-1 DNA; CA-RNA CD4-cell associated HIV-1



Supplementary Figure 3. Differences in WBC percentages between PLHIV and healthy individuals (previous page). Differences in WBC percentages between PLHIV (n=211) and healthy individuals (n=56) ordered in an hierarchical tree like structure in whole blood (**A**) and peripheral blood mononuclear cells (**B**). Inverse-rank transformed data were analyzed using linear regression and adjusted for age, sex, sampling time, and season. For color coding of the FDR-adjusted p-values see legend.



Supplementary Figure 4. Differences in Th17/Treg ratios with different markers. (**A**) Th17/Treg ratio based in which Tregs were identified using FoxP3 and Helios. (**B**) Th17/Treg ratio based in which Tregs were identified using CD25 and CD127. Results are comparable with results reported in Figure 1B Th17: T-helper 17 (Mem Tc CCR6+ CXCR3-CCR4+); Treg: regulatory T cell

Supplementary table 1 10-color flow cytometry panels

	Flow channel	FL1	FL2	FL3	FL4	FL5	FL6	FL7	FL8	FL9	FL10
Panel	Fluorochrome	FITC	PE	ECD	PE-Cy5.5	PC7	APC	APC-AF700	APC-AF750	PB	KO
LMI 1	mAb	CD16	HLA-DR	CD14	CD4	CD25	CD56	CD8	CD19	CD3	CD45
General	Clone	3G8	immu-357	UCHT1	1388.2	M-A251	N901	B9.11	J3-119	UCHT1	J33
Whole blood	Distributor	Coulter	Coulter	Coulter	Coulter	BD	Coulter	Coulter	Coulter	Coulter	Coulter
	Fluorochrome	FITC	PE	ECD	PE-Cy5.5	PC7	APC	APC-AF700	APC-AF750	PB	KO
LMI 2	mAb	CD45RA	CD3	CD45RO	CD27	CD25	CD56	CD127	CD8	CD4	CD45
T cell	Clone	ALB11	UCHT1	UCHL1	1A4CD27	M-A251	N901	R34.34	B9,11	13B8.2	J33
Whole blood	Distributor	Coulter	Coulter	Coulter	Coulter	BD	Coulter	Coulter	Coulter	Coulter	Coulter
	Fluorochrome	FITC	PE	ECD	PE-Cy5.5	PC7	APC	APC-AF700	APC-AF750	PB	KO
LMI 3	mAb	IgD	IgM	CD3	CD27	CD38	CD24	CD5	CD19	CD20	CD45
B cell	Clone	ADB6	SA-DA4	UCHT1	1A4CD27	LS198-4-3	ALB9	BL1a	J3-119	B9E9	J33
Whole blood	Distributor	Coulter	Coulter	Coulter	Coulter	Coulter	Coulter	Coulter	Coulter	Coulter	Coulter
	Fluorochrome	FITC	PE	ECD	PE-Cy5.5	PC7	AF674	APC-AF700	APC-AF750	e450	KO
LMI 4	mAb	KI67 (ic)	HLA-DR	CD45RA	CD4	CD25	Helios (ic)	CD127	CD8	FoxP3 (ic)	CD45
T cell / Treg	Clone	B56	immu-357	2H4LDH11LD89	1388.2	M-A251	22F6	R34.34	B9.11	PCH101	J33
PBMC	Distributor	BD	Coulter	Coulter	Coulter	BD	Biolegend	Coulter	Coulter	eBioscience	Coulter
	Fluorochrome	FITC	PE	ECD	PerCp5.5	PC7	APC	AF700	APC-pc7*	BV421*	KO
LMI 5	mAb	CD45RA	CD196	CD8	CD183	CD194	CD25	CD4	CD195	CD197	CD45
Chemokine	Clone	ALB11	11A9	SFCI21Thy	G025H7	1G1	2A3	RPA-T4	2D7	G043H7	J33
Whole blood	Distributor	Coulter	BD	Coulter	Biolegend	BD	BD	eBioscience	BD	Biolegend	Coulter

Samples were analyzed by a 3-laser Navios (Beckman Coulter).
mAb: monoclonal antibody; IC: intracellular staining; PBMC: peripheral blood mononuclear cells
* Excluded from analysis in this paper

Supplementary table 2. Stimulation scheme for the *ex vivo* cytokine production assays

Stimulus	Final Concentration	Strain/Manufacture
RPMI + serum	10% human serum	Life technologies
Staphylococcus aureus	1 x 10^6/mL	ATCC 29213 (in house)
Cryptococcus gattii	1 x 10^7/mL	A1M-R265, AFLP type 6 (clinical isolate)
Candida albicans conidiae	1 x 10^6/mL	UC820 (in house)
Candida albicans hyphae	1 x 10^6/mL	UC820 (in house)
Streptococcus pneumonia	1 x 10^6/mL	TIGR4
Mycobacterium tuberculosis	1 µg/mL	H37Rv (in house)
Imiquimod (TLR7 ligand)	2.5 ug/mL	Invivogen

PBMCs: peripheral blood mononuclear cells; TLR: Toll-like receptor.
*All 7 day PBMC stimulation experiments were supplemented with 10% human pool serum.

Supplementary table 3. Primers and probes to determine CA-DNA and CA-RNA

Assay	Oligo	Modification	Sequence
Total HIV-1 DNA	Forward		5'-GCCTCAATAAAGCTTGCC-3'
and cell-associated HIV-1 RNA	Reverse		5'-GGCGCCACTGCTAGAGATTTT-3'
	Probe	6FAM/MGB Eclipse	5'-AAGTRGTGTGCCC-3'
Reference gene RPP30	Forward		5'-AGATTTGGACCTGCGAGCG-3'
	Reverse		5'-GAGCGGCTGTCTCCACAAGT-3'
	Probe	HEX/ZEN/3' IBFQ	5'-TTCTGACCTGAAGGCTCTGCGCG-3'
Reference gene B2M	Forward		5'-TGTCGCGATGGATGAAACCCAGA-3'
	Reverse		5'-TGCTCGCGCTACTCTCTCTTT-3'
Reference gene ACTB	Forward		5'-CCGATCCACACGGAGTACTT-3'
	Reverse		5'-TGGACATCCGCAAAGACCTG-3'
Reference gene GAPDH	Forward		5'-GAACATGCTGATGGCATTTC-3'
	Reverse		5'-GAAGGTGAAGGTCGGAGTC-3'

Supplementary Methods

HIV-1 DNA and cell-assicioated HIV-1 RNA quantification in CD4+ T cells

See Methods for the description of the HIV-1 DNA and RNA extraction. Before PCR amplification, 8.65µl of genomic DNA was restricted by EcoRI (Promega) in a total volume of 10µl restriction digest for a minimum of 1 hour at RT. Total HIV-1 DNA and HIV-1 RNA were measured by adding respectively 2µl and 4µl in triplicates to ddPCR mix containing 10µl 2x ddPCR Supermix for Probes, 500nM primers and 300 nM probe (Table X). DNA was amplified by PCR with an initial denaturation step of 10min at 95°C, followed by a denaturation step for 30 sec at 95°C, an annealing/elongation step for 1 min at 56°C for 40 cycles, and a final step of 10min at 98°C. Total HIV-1 DNA was normalized by measuring the reference gene RPP30 (Table X) in duplicate by ddPCR and expressed per million PBMCs. Droplets were read by QX200 droplet reader (Bio-Rad) and automatic threshold setting was done using ddpcRquant software[26]. For HIV-1 RNA normalization, three reference genes per patient, B2M, ACTB and GAPDH (Supplementary Table S3) were measured with LightCycler 480 SYBR Green I Master mix. HIV-1 RNA copies were divided by the geometric mean of the reference genes and expressed per million PBMCs.

Immunophenotyping

Staining

For surface staining, cells were incubated in 25 µl surface staining master mix for 20 min at RT and washed twice in PBS + 0.2% BSA. Before acquisition, whole blood-derived cells were resuspended in 100 µl PBS + 0.2% BSA. For intracellular staining, surface-stained PBMCs were fixed and permeabilized using Fixation/Permeabilization solution (eBioscience, Vienna, Austria) for 30 min at 4°C protected from light. After washing the cells twice in permeabilization buffer (eBioscience, Vienna, Austria), cells were stained with 25 µl intracellular staining master mix for 30 min at 4°C protected from light. After a second washing step with permeabilization buffer, cells were resuspended in 100 µl PBS + 0.2% BSA for acquisition.

Flow cytometry

For each panel, single cells within the leukocyte ($CD45^+$) population were identified by plotting the forward scatter (FSC) against FSC time of flight (FSC TOF), followed by characterization of the major myeloid or lymphoid lineages [17]. The absolute number of WBC per ml of blood determined by the Sysmex XN-450 hematology analyzer was used to calculate absolute numbers of leukocyte ($CD45^+$) cell subsets as measured by flow cytometry.

Panel 1 (general) identified granulocytes, lymphocytes (both by FSC, side scatter [SSC]) and monocytes ($CD14^+$). Lymphocytes were further characterized into T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), NKT cells ($CD3^+CD56^+$), and B cells ($CD19^+HLA^-DR^+$). Cell subsets were determined for T cells (CD4 and CD8), NK cells (CD56 and CD16) and monocytes (CD14 and CD16). Panel 2 (T cell) covered $CD4^+$ regulatory T cells (Treg; $CD4^+CD25^+CD127$ low) and maturation stages of $CD4^+$ and $CD8^+$ T cells (using CD45RA/CD27 and CD45RO/CD27). Panel 3 (B cell) explored CD19+ B cell maturation stages by the expression of IgM/IgD and/or CD24/CD38 and B cell subsets by differential CD19/CD20 and IgD/CD5 expression, as previously described [17]. In panel 4 (intracellular T cell/Treg), the major T cell populations (CD4, CD8 and Treg) were identified and analyzed for proliferation status by intracellular Ki67 expression. Tregs ($CD4^+CD25^+CD127$low $FoxP3^+$) were analyzed for expression of Helios, CD45RA, and HLA-DR. Absolute cell counts in this panel were calculated as described above by subtracting the granulocyte number (determined by panel 1) from the PBMC WBC counts (Sysmex XN-450). In panel 5 (chemokine), monocytes and T cell populations (CD8, CD4 including memory T cells [Mem Tc; $CD45RA^-CD25^-$] and Treg [$CD45RA^-CD25^{++}$]) were analyzed for the expression of different cc-chemokine receptors (CCR): CXCR3 (CD183), CCR4 (CD194), and CCR6 (CD196).

Statistical methods

Linear regression analysis

The following regression formula was used:

$$Y1 \sim X1 + age + sex + sin(2 * pi * numDaysFromJan2015/365) + cos(2 * pi * numDaysFromJan2015/365) + numDaysFromJan2015$$

where sin(2 * pi * numDaysFromJan2015/365) and cos(2 * pi * numDaysFromJan2015/365) capture seasonality patterns with a periodicity of one year[19]. The cell count data showed some slight drift over time which was corrected using the linear term *numDaysFromJan2015*, indicating after how many days after Jan 1st 2015 the sample was collected.

Analysis of correlation structures between 200HIV and 500FG

Correlations were performed on absolute WBC counts instead of WBC proportions.
To properly ascertain cell count correlations, we first corrected the IRT-normalized cell counts for age, sex, and seasonal effects, by regression out these factor using a model similar to the one described above. We calculated inter-cohort differences in strength of cell count associations using the following method. First, we defined the test statistic:

$$D\_real = correlation\_X1\_Y1\_cohort1 - correlation\_X1\_Y1\_cohort2 = C1 - C2$$

With hypotheses: $H0: c1 = c2$ and $H1: c1 \neq c2$

where X1 and Y1 refer to the different cell types. Within each cohort the ID-labels were shuffled 10,000 times for each WBC type and the correlation between the cell types in both cohorts were calculated for all 10,000 permutations, yielding 10,000 unique correlation differences.

$$D\_permuted = correlation\_X1\_Y1\_cohort1 - correlation\_X1\_Y1\_cohort2$$

P-values were obtained using two different methods. First, P-values were calculated by dividing the number of times the absolute shuffled test statistics were higher than the real absolute test statistic.

$$(number\ of\ times\ abs(D\_permuted) >= abs(D\_real)) + 1)/number\ of\ permutations$$

Second, p-values were obtained by applying a half-normal distribution to the absolute D_ permuted values. P-values were calculated out of the percentage of the area with absolute permuted values higher than the real correlation. This second approach yielded more precise p-values without increasing the number of permutations (as for the discrete permutation analysis the lowest possible p-value is *1/number of permutations)*. Here, the p-values from the fitted distribution are reported. Using the p-values from the discrete method (first method) showed very similar pattern, and therefore selecting one method over the other does not change the overall conclusions.

Chapter 4

Clonal hematopoiesis is associated with low CD4 nadir and increased residual HIV transcriptional activity in virally suppressed individuals with HIV

Wouter van der Heijden[#], Rosanne van Deuren[#], Lisa van de Wijer, Inge van den Munckhof, Marloes Steehouwer , Niels Riksen, Mihai Netea, Quirijn de Mast, Linos Vandekerckhove, Richarda de Voer, Andre van der Ven, Alexander Hoischen

[#] Equal contribution

The Journal of Infectious Diseases, in press

Abstract

Clonal hematopoiesis, a common age-related phenomenon marked by expansion of cells with clonal hematopoiesis driver mutations, has been associated with all-cause mortality, cancer and cardiovascular disease. People with HIV (PLHIV) are at risk for non-AIDS related comorbidities such as atherosclerotic cardiovascular disease and cancer. In a cross-sectional cohort study, we compared clonal hematopoiesis prevalence in PLHIV on stable antiretroviral therapy, with prevalence in a cohort of overweight individuals and a cohort of age- and sex-matched population controls. The prevalence of clonal hematopoiesis adjusted for age was increased and clone size was larger in PLHIV compared to population controls. Clonal hematopoiesis is associated with low CD4 nadir, increased residual HIV-1 transcriptional activity and coagulation factors in PLHIV. Future studies on the effect of clonal hematopoiesis on the HIV reservoir, and non-AIDS related comorbidities are warranted.

Introduction

People with HIV (PLHIV) are at risk for non-AIDS related comorbidities such as atherosclerotic cardiovascular disease (CVD) and cancer[1,2]. This risk is associated with persistent inflammation, increased coagulation, accelerated aging, CD4 nadir and certain antiretroviral drugs[1].

Recently, clonal hematopoiesis (CH), a common age-related phenomenon marked by expansion of cells with clonal hematopoiesis driver mutations, has been associated with all-cause mortality, cancer and CVD[3-5]. Aside from age, chronic infection[6] and inflammation[7,8] have also been implicated in the development of clonal hematopoiesis. As PLHIV experience persistent inflammation and are at increased risk for accelerated aging and CVD[9,10], a role for CH in PLHIV is therefore hypothesized. Indeed, a recent study observed increased prevalence of CH as detected by whole exome sequencing (WES) in PLHIV as compared to controls[11]. However, associations with HIV-related factors and risk factors of CVD and cancer in PLHIV, such as the viral reservoir, CD4 nadir or inflammation were not shown. As the clinical course and treatment of HIV infection and CH possibly converge on hematopoietic stem cell biology, a potential correlation deserves investigation. Additionally, alike many other large-scale CH studies, the study used WES and as such could only detect mutations of considerable size (≥2%, also called clonal hematopoiesis of indeterminate potential (CHIP) mutations), whereas the use of targeted sequencing techniques improves the sensitivity for the detection of smaller CH driver mutations[12].

The aim of our study was to determine clonal hematopoiesis prevalence in PLHIV as compared to controls, and to assess possible associations with HIV-related clinical parameters, and markers of HIV reservoir, coagulation and inflammation.

Materials and Methods

Study subjects

Samples were obtained from the 200HIV cohort (PLHIV cases, n = 219)[13] on antiretroviral therapy (cART) with plasma HIV-RNA <200copies/mL, 300-Obese cohort (HIV-uninfected, overweight controls (BMI>27kg/m^2), n = 302)[14], and Nijmegen Biomedical Study (NBS, HIV-uninfected, population controls, n = 437)[15]. The 200HIV and 300-Obese cohort are both part of the Human Functional Genomics Project (www.humanfunctionalgenomicsproject.org) enrolled at the Radboud University Medical Center, Nijmegen, the Netherlands. These studies were approved by the local ethics committee (CMO Arnhem-Nijmegen, 200HIV: NL3235709110; 300-Obese: NL4684609113) and conducted in accordance to the Declarations of Helsinki. Samples for DNA extraction and plasma measurement were obtained after written informed consent. Clinical data were extracted from the electronic medical records and/or from the Dutch HIV registry (Stichting HIV-monitoring). The NBS is a population-based study of 9,350 individuals, based on age- and sex-stratified random sample from the register of municipality of Nijmegen, the Netherlands[15].

Clonal Hematopoiesis mutation identification

Clonal Hematopoiesis (CH) mutations were analyzed in DNA isolated from whole blood in PLHIV and 300-Obese cohorts, using ultra-sensitive single-molecule Molecular Inversion Probe (smMIP) sequencing, as previously described[16]. In short, a total of 300 MIP-probes were designed covering CH-related hotspots in 24 genes, including *ASXL1*, *TET2* and *DNMT3A* (Supplementary Table S1 and Supplementary Table S2). In addition, we selected 437 age- and sex-matched population control samples, and used the previously generated sequencing data for CH mutation identification, but due to subtle technical (probe density) differences excluded *DNMT3A* mutations from this direct comparison [15]. For each individual, two technical (PCR) replicates were sequenced, after which two independent data processing strategies were applied followed up by a targeted quality control (Supplementary Figure S1). Raw sequencing data were converted to fastQ-files, which were 1) aligned to the reference genome (hg19) using BWA-MEM[17] and 2) imported into the commercially available NGS software package Sequence Pilot (JSI Medical Systems), using the optimized smMIP analysis module as described previously[18,19]. The latter allows for a consensus calling per probe, enabling somatic calls down to 0.001% (depending on locus specific coverage) by using a majority vote of Unique Molecular Identifier duplicates. The resulting variant calls were then subjected to a stringent quality filtering pipeline (Supplementary Figure S1C), in which (likely) false positive calls were excluded. The final variant allele frequency was calculated using samtools mpileup[20] on the aligned bamfiles from 1). PLHIV and 300-Obese samples with an average coverage over the entire panel, and population control samples over all non *DNMT3A* genes, of >500x were included for analysis. We classified all detected CH mutations in two categories: 1) large clones – CH mutations with a variant allele frequency ≥2%, an arbitrary threshold established in the current literature historically chosen for methodological reasons[21] and 2) small clones – CH mutations with a variant allele frequency <2%.

Circulating factors

Circulating factors of inflammation IL-18, high-sensitivity C-reactive protein (hsCRP), sCD14 and sCD163 were measured using ELISA (Duoset or Quantikine, R&D systems). D-dimer was measured by ELISA according manufacturer's instructions (Abcam, Cambridge, UK). IL-6, TNFα, IL-10 and IL-1Ra in serum were measured using Simple Plex Cartridges (Protein Simple, R&D systems, USA). All assays were performed according to manufacturer's recommendations. Von Willebrand factor (vWF) concentrations were performed with an in-house sandwich ELISA assay (DAKO, Agilent, Santa Clara, USA)[22].

HIV-1 reservoir quantification

HIV-1 CA-DNA and CA-RNA in CD4+ T cells isolated using EasySep Human CD4+ T Cell Isolation Kit (Stemcell technologies, Vancouver, Canada), were measured in triplicate by droplet digital PCR (ddPCR QX200 – Bio-Rad) as described previously[23]. Genomic DNA was extracted using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol with an additional step of adding 75µl elution buffer on the column heated at 56°C for 10 min. CA-RNA was extracted using the Innuprep RNA kit (Westburg, Leusden, The Netherlands). RNA was reversely transcribed to cDNA by qScript cDNA SuperMix (Quantabio, Beverly, MA, USA). Before PCR amplification, genomic DNA was restricted by EcoRI (Promega)[23]. Total HIV-1 DNA measurements were normalized by measuring the reference gene RPP30 in duplicate by ddPCR and expressed per million CD4+ cells. CA-RNA was normalized using three reference genes, (B2M, ACTB and GADPH) determined by LightCycler 480 SYBR Green I Master mix. HIV-1 RNA copies were divided by the geometric mean of the reference genes and expressed per million CD4+ cells. Droplet classification and absolute quantification was performed using the ddpcRquant analysis tool with standard settings[24]. Primers and probes are shown in Supplementary Table S3.

Statistical analysis

All analyses were performed in R version 3.6.1 (R Core Team, CRAN-project). P-values <0.05 were considered statistically significant. General characteristics were compared by means of Wilcoxon-rank sum tests for continuous parameters and Chi-square tests for categorical parameters. In addition, we computed standardized mean differences where appropriate using the R-package stddiff.

CH mutation prevalence in PLHIV versus 300-Obese was assessed first by means of Chi-square tests, and second in a logistic regression model correcting for the known risk factor age and cohort (a variable indicating whether an individual is in the PLHIV or 300-Obese). Prevalence of non *DNMT3A* CH mutations in PLHIV versus population controls was assessed in the same way. Logistic model fit was evaluated by means of a givitiCalibrationBelt-plot using the package givitiR, and are shown in Supplementary Figure S2.

In order to assess whether different mutational processes contribute to CH mutations in PLHIV as compared to controls, we performed a mutational signature analysis studying all 48 single base substitutions (SBS) in their three-nucleotide context (see Supplementary Table S4)[25]. Fitting all identified somatic mutations into existing SBS signatures for which mutational processes of both endogenous and exogenous origin have been characterized, may allow the identification of differential mutation processes for different sets of mutations. The contribution of the single base substitution (SBS) signatures was inferred using the R package DeconstructSigs[26] and Mutational Signatures V3.1 available at the Catalogue of Somatic Mutations in Cancer (COSMIC release v91, June 2020).

Within PLHIV differences in categorical parameters were assessed by means of Chi-square tests, whereas continuous parameters by means of Wilcoxon-rank sum tests. In addition, to explore possible factors that are correlated with clonal hematopoiesis in PLHIV, we performed stepwise logistic regression with a selection of clinically relevant parameters as independent variables, CH mutation prevalence as dependent variable using the stepAIC() function with direction="both" from the MASS package.

All figures were likewise generated in R using a variety of packages (dplyr, reshape2, ggplot2, ggpubr, tidyverse, ggpmisc, rcompanion, ggbeeswarm), after which they were optimized in Adobe Illustrator version 23.1.1 (Adobe Inc.).

Results

A total of 217 PLHIV on combination antiretroviral therapy, 297 uninfected 300-Obese control, and 399 age- and sex-matched population control individuals passed our quality control and coverage threshold of 500x (Supplementary Figure S3), and were included in our analyses. The age of PLHIV individuals ranged from 24 to 74, with an average age of 51.3 years, and the majority were male (n = 199, 91.7%). HIV-uninfected overweight controls (300-Obese) were generally older, (age range 54-81, average age 67.1 years), and sex was more evenly distributed (male n = 134, 45.1%). The age and sex distribution of our included population controls was comparable to PLHIV individuals, with an age ranging from 24 to 69 and an average of 51.9 years, and majority of individuals were male (n = 365, 91.5%).

CH mutation prevalence in PLHIV versus uninfected controls

We identified 51 candidate CH mutations in 46 PLHIV individuals (21.2%), five individuals presenting with two different mutations. General characteristics for PLHIV CH mutation carriers versus non-carriers are shown in Table 1. The variant allele frequency of all CH mutations ranged from 0.05% to 32.94%, with a median clone size of 1.01%. Out of 51 mutations, 20 (39.2%) were categorized as large clones (≥2%), highlighting the sensitivity of our assay to detect somatic mutations below the arbitrary allele frequency cutoff for CHIP[3,15]. For a complete list of all identified candidate CH mutations see Supplementary Table S5.

In our HIV-uninfected overweight controls (300-Obese), we identified 110 candidate CH mutations in 85 individuals (28.6%). We were unable to detect a significant difference in either CH mutations or CHIP prevalence between PLHIV and overweight controls (Supplementary Table S6). Yet, after correcting for the most important known risk factor age in a logistic model, we identified that the probability of CHIP in PLHIV is significantly higher as compared to HIV-uninfected overweight controls (OR cohort = 2.21 95% CI [1.03-4.77]), an effect that did not reach statistical significance when smaller clones (variant allele frequency <2%) were included (Supplementary Table S7). Most observed clones in PLHIV were mutations in *DNMT3A*, representing roughly half of the CH mutations per age category (Figure 1A). Comparing the gene occurrence in PLHIV age ≥55 to controls age ≥55, we observed that the proportion of CH mutations in genes other than *DNMT3A*, specifically *JAK2*, *STAT3*, and *TP53*, was larger in PLHIV (Figure 1B), whereas on average CH mutation size was not different (Figure 1C). Considering only non-*DNMT3A* mutations in our age- and sex-matched population controls, we identified 55 candidate CH mutations in 42 individuals (10.5%), as compared to 25 in 23 individuals (10.6%) in PLHIV. Even though there was no significant difference in raw prevalence (p=1), logistic regression revealed that the effect of age on CH mutation prevalence was significantly smaller in PLHIV as compared to population controls, suggesting that non-age related factor(s) "drive(s)" the prevalence of CH in PLHIV (Supplementary Table S7). Finally, non-*DNMT3A* CH mutations were significantly larger in size in PLHIV as compared to population controls (p=0.004, Figure 1D).

Table 1. General characteristics of PLHIV without clonal hematopoiesis (CH) mutations (non-carriers) as compared to PLHIV in which one or more CH mutations were identified (CHDM-carriers).

	non-carriers (n=171)	CH-carriers (n=46)	p
Sex Female - n (%)	13 (7.6%)	5 (10.9%)	0.68
Age - mean [IQR] (years)	50.0 [44.0-58.0]	57.0 [48.0-62.2]	0.006
BMI - mean [IQR] (kg/m2)	24.2 [22.0-26.5]	23.7 [22.4-25.8]	0.48
Known duration of HIV infection - mean [IQR] (years)	7.5 [4.7-12.7]	10.6 [6.8-16.9]	0.03
Way of transmission			0.44
Heterosexual - n(%)	8 (4.7%)	1 (2.2%)	
IDU - n(%)	2 (1.2%)	1 (2.2%)	
MSM - n(%)	132 (77.2%)	32 (69.6%)	
Other/unknown - n(%)	29 (17.0%)	12 (26.1%)	
CD4 nadir - mean [IQR] (cells/uL)	270.0 [150.0-380.0]	195.0 [72.5-290.0]	0.001
Current CD4 T-cell count- mean [IQR] (cells/uL)	660.0 [490.0-805.0]	635.0 [472.5-755.0]	0.58
Current CD4/CD8 T-cell ratio - [IQR]	0.8 [0.6-1.1]	0.8 [0.6-1.2]	0.37
Zenith HIV-RNA - median [IQR] (copies/mL)	100000.0 [60000.0-400000.0]	70000.0 [30281.5-222250.0]	0.04
HIV-RNA >50copies/mL <1 yr prior to inclusion, n (%)	14 (8.2%)	9 (19.6%)	0.05
cART duration - mean [IQR] (years)	6.1 [3.7-10.2]	9.1 [4.4-16.8]	0.05
ARV classes			
NRTI - n (%)	165 (96.5%)	44 (95.7%)	1
NtRTI - n (%)	79 (46.2%)	23 (50.0%)	0.77
NNRTI - n (%)	50 (29.2%)	13 (28.3%)	1
PI - n (%)	21 (12.3%)	11 (23.9%)	0.08
AZT ever - n (%)	39 (23.2%)	18 (39.1%)	0.048
Cardiovascular risk factors No - n (%)	45 (26.3%)	6 (13.0%)	0.09
MHCVD Yes - n (%)	42 (24.6%)	16 (34.8%)	0.23
Prior MI/CVA Yes - n (%)	11 (6.4%)	5 (10.9%)	0.48
Active smoking - n (%)	44 (25.7%)	17 (37.0%)	0.19
Packyears	12.0 [0.0, 27.0]	18.5 [1.9, 35.1]	0.18
Hypercholesterolemia - n (%)	45 (26.3%)	13 (28.3%)	0.94
Hypertension - n (%)	33 (19.3%)	10 (21.7%)	0.87
Diabetes Mellitus - n (%)	9 (5.3%)	1 (2.2%)	0.62
Family history CVD 1st degree - n (%)	81 (47.4%)	26 (56.5%)	0.35

BMI: body mass index. IDU: intravenous drug-use. MSM: men who have sex with men. cART: combination antiretroviral therapy. NRTI: Nucleoside reverse transcriptase inhibitor. NtRTI: nucleotide reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor. INSTI: integrase inhibitor. AZT: zidovudine. MI: myocardial infarction. CVA: cerebrovascular accident. Wilcoxon-rank sum test or Chi-square test P-value is depicted where applicable.

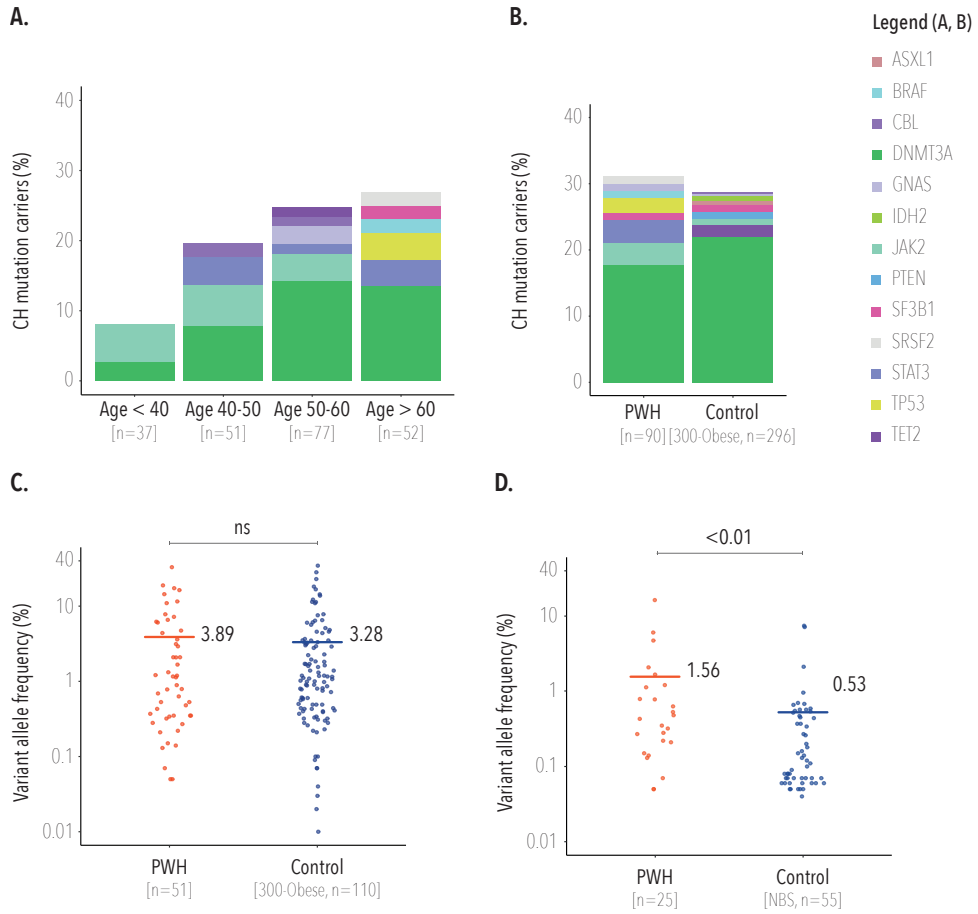


Figure 1. Clonal Hematopoiesis (CH) mutations in people with HIV (PLHIV), HIV-uninfected overweight controls (300-Obese), and HIV-uninfected age- and sex-matched population controls (NBS). (**A**) CH mutation prevalence is increased with age in PLHIV. The stacked bars represent the percentage of PLHIV carrying a CH mutation per age category (the CH mutation with highest variant allele frequency is plotted for individuals carrying multiple mutations). (**B**) The proportion of CH mutations in genes other than DNMT3A in PLHIV age ≥55 (n = 90) is larger as compared to HIV-uninfected overweight controls (n = 296). (**C**) CH mutation size is non-significantly increased in PLHIV as compared to HIV-uninfected overweight controls. (**D**) CH mutation size is significantly larger in PLHIV as compared to population controls. In (**C**) and (**D**) each dot represents a CH mutation (the number of mutations is annotated on the x-axis and the size on the y-axis), with a horizontal line and number to the right indicating the mean CH mutation size and the Wilcoxon-rank sum test P-value annotated at the top.

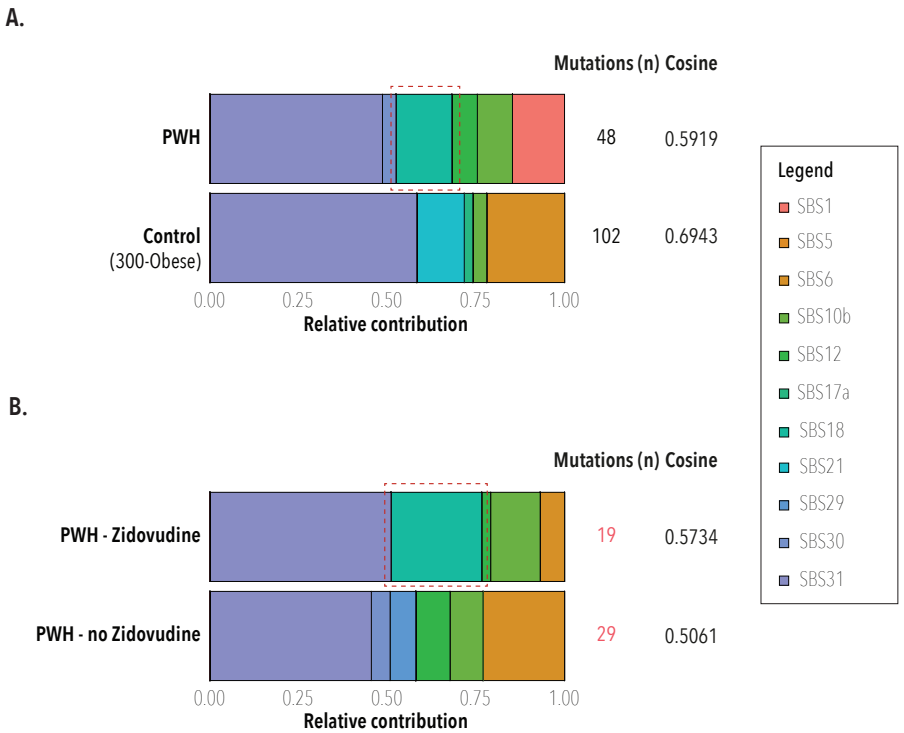


Figure 2. PLHIV present with a distinct mutational signature profile. (**A**) shows the contribution of different SBS signatures to Clonal Hematopoiesis (CH) mutations in PLHIV (top) and HIV-uninfected overweight controls (bottom). SBS1 and SBS18 contribute uniquely to CH mutations in PLHIV, whereas SBS6 and SBS21 are unique to controls. For raw results see Supplementary Table S8A. (**B**) shows that SBS18, a signature predominantly characterized by C>A mutations that is involved in reactive oxygen species (ROS) production, uniquely contribute to CH mutations in PLHIV with prior exposure to AZTs. For raw results see Supplementary Table S8B. Proposed etiology of SBS signatures: SBS1 - Spontaneous deamination of 5-methylcytosine (clock-like signature); SBS5 - Unknown (clock-like signature); SBS6 - Defective DNA mismatch repair; SBS10b - Polymerase epsilon exonuclease domain mutations; SBS12 - Unknown; SBS17a - Unknown; SBS18 - Damage by reactive oxygen species; SBS21 - Defective DNA mismatch repair; SBS29 - Tobacco chewing; SBS30 - Defective DNA base excision repair due to NTHL1 mutations; SBS31 - Platinum chemotherapy treatment. Annotation: Mutations (n) shows the number of CH mutations analyzed in each group; Cosine represents the cosine similarity value (range 0-1) - a correction applied on mutations prior to fitting to SBS signatures - values <0.8 are considered low.

CH mutations in PLHIV individuals are possibly driven by different mutational processes

To understand the mutational processes contributing to CH mutations, we explored mutational signatures, *i.e.* base substitutions in a tri-nucleotide context[25]. Our mutational signature analysis identified the signatures SBS1 (clock-like signature) and SBS18 (ROS-signature) to contribute uniquely to CH mutations in PLHIV, whereas SBS6 and SBS21 (both involved in DNA mismatch repair) contribute uniquely to CH mutations in HIV-uninfected overweight controls (Figure 2A). Remarkably, SBS18, a signature predominantly characterized by C>A mutations was likewise identified to contribute to CH mutations in PLHIV with prior exposure to AZT, whereas it was absent in unexposed individuals (red dashed square in Figure 2A and Figure 2B). However, as the number of CH mutations in this last sub-group analysis is low, the corresponding cosine value indicates these results should be interpreted with caution.

Low CD4 nadir, markers of coagulation and HIV reservoir are associated with CH mutation prevalence in PLHIV

Subsequently, we explored the clinical correlates of PLHIV with their CH mutation carrier status. In these exploratory analyses, CH mutation carriers were older (p=0.006, Table 1), duration of known HIV infection was longer (p=0.026, Table 1), and CD4 nadir was lower (p=0.001; Figure 3A). There was no difference in CH mutation prevalence with regard to combination antiretroviral therapy (cART) regimens, CD4/CD8 T-cell ratio or current CD4 T-cell count. In a logistic regression model, older age (OR (+5yr): 1.21 [CI:1.01-1.45], p=0.04), lower CD4 nadir (OR (+50cells/mm²): 0.794 [CI:0.687-0.907], p=0.001), increased CD4/CD8 ratio (OR: 2.85 [CI:1.33-6.39], p=0.008) were independently correlated to CH mutation prevalence, whereas HIV-duration and current CD4 T cell count were not (Supplementary Table S7).

PLHIV with a CH mutation were more likely to have at least once a viral load of >50 copies/mL prior to study visit (19.6% vs 8.2%, p=0.052), suggesting a more active HIV reservoir. In virally suppressed PLHIV, the HIV-1 CA-DNA roughly equals total integrated proviral DNA, while HIV-1 CA-RNA is associated with HIV-1 transcriptional activity[27]. Although these parameters of HIV reservoir strongly intercorrelate, HIV CA-RNA was increased in CH mutation carriers and HIV CA-DNA was not (Figure 3B). The discrepancy between CA-RNA and CA-DNA suggests an effect on transcriptional activity[27]. As a result, PLHIV with viral load between 50 and 200 copies/ml (viral blip) 1 year prior to study visit (10.6%) had higher levels of CA-RNA (p<0.01), but no differences in CA-DNA. Although the CA-RNA difference disappeared after correction for CD4 nadir and age (Supplemental Table S7), the ratio of HIV-1 CA-RNA to CA-DNA, which represent relative viral transcription level[27], was increased in CH mutation carriers (OR: 5.56 [CI:1.77-45.97], p=0.017; Supplementary Table S7), independent of CD4 nadir, age and CD4/CD8 T cell ratio.

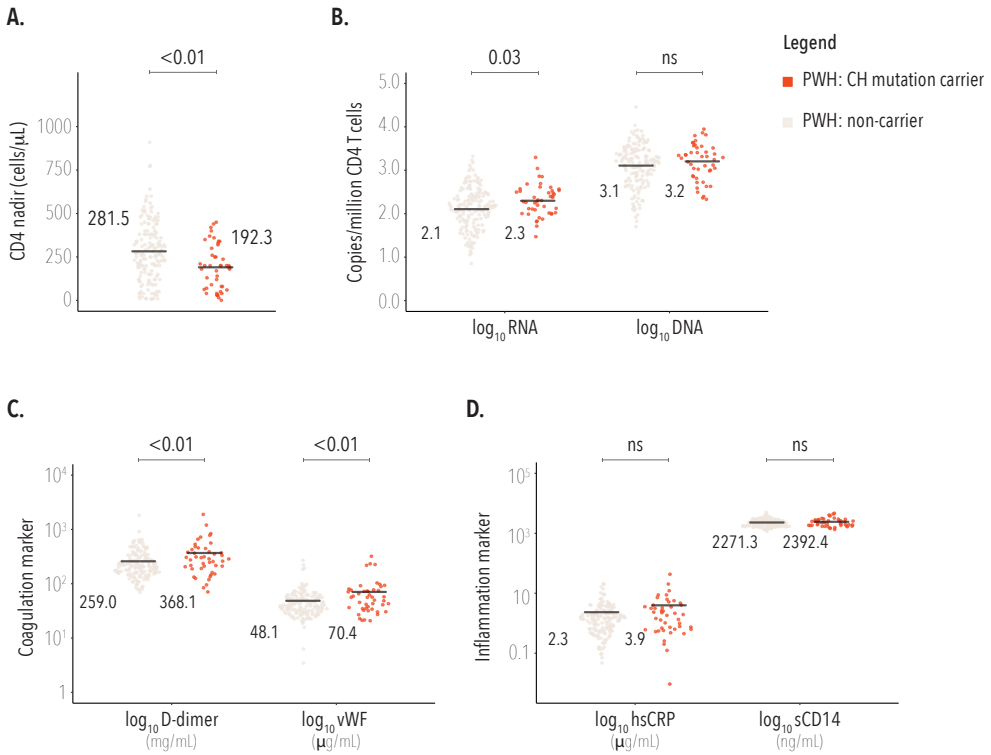


Figure 3. PLHIV Clonal Hematopoiesis (CH) mutation carriers present with significantly lower CD4 nadir, and higher cell-associated HIV-RNA and coagulation markers. (**A**) Carriers (n = 46) present with significantly lower CD4 nadir as compared to non-carriers (n = 171). (**B**) Carriers (n = 45) show significantly increased RNA levels as compared to non-carriers (n = 169), and non-significantly increased DNA levels as compared to non-carriers (n = 167). (**C**) Carriers (n = 46) present with significantly increased coagulation markers D-dimer and von Willebrand Factor (vWF) as compared to non-carriers (n = 171). (**D**) Carriers (n = 45) show comparable high-sensitivity C-reactive protein (hsCRP) as compared to non-carriers (n = 167) and carriers (n = 46) show comparable soluble CD14 (sCD14) levels as compared to non-carriers (n = 170). In each plot the mean of non-carriers and CH mutation carriers is annotated with a horizontal bar and value, at the top the Wilcoxon-rank sum test P-value is depicted.

Additionally, we studied associations between CH mutation carriership, coagulation and inflammation. No correlation was found with circulating inflammation markers (hsCRP, sCD14, sCD163, IL-6), while coagulation markers (d-dimer and vWF) were increased in CH mutation carriers (Figure 3C, 3D and Supplementary Figure S4), independent of age, CD4 nadir and CD4/CD8 T-cell ratio (vWF: OR (+5000ng/mL): 1.053 [CI:1.014-1.097], p=0.009; d-dimer: OR (+50mg/mL): 1.076 [CI:1.004-1.163], p=0.044; Supplementary Table S7).

Discussion

In the current study, we show that clonal hematopoiesis driver mutations in PWH on long-term successful cART are common and are independently associated with older age, low CD4 nadir, increased CD4/CD8 T-cell ratio, as well as with markers of coagulation. Furthermore, we show that clonal hematopoiesis is linked to increased relative HIV-1 transcription level (*e.g.* increased HIV-1 CA-RNA to HIV-1 CA-DNA ratio).

Age is a known clonal hematopoiesis predictor in the general population[3,4,16], as well as in the current study, supporting the validity of our data. CH may be important in PWH as their presence has been associated with hematological (pre)malignancies, as well as with CVD and all-cause mortality in HIV uninfected individuals[3]. Recently, a large-scale clonal hematopoiesis study by Bick *et al.* reported increased prevalence of large clones, or CHIP, in PWH as compared to control individuals[11]. In line with this, the probability of large clones adjusted for age was increased in PWH as compared to HIV-uninfected overweight controls, and the probability of all non-*DNMT3A* clones (small and large) was intriguingly driven by something other than age in PWH as compared to population controls in present study. Consistent with the latter, and the hypothesis that time is needed for a clone to grow, clone size was significantly increased in PWH as compared to age- and sex-matched population controls. The absence of this in our PWH versus overweight controls comparison is most likely due to the differences in age and BMI, as we have recently shown that clone size may be increased in obese individuals [16].

Our observation that something other than age drives CH prevalence in PWH, was substantiated by the fact that the mutational processes underlying the identified CH mutations may be different between PWH and controls. Only in PWH, a ROS mutational signature could be assigned to some of the observed CH mutations. ROS production is indeed increased in PWH and, interestingly, has been linked to nucleoside reverse transcriptase inhibitor (NRTI) induced mitochondrial toxicity[3,4]. The enhanced ROS-signature found in CH mutations from PWH compared to HIV-uninfected overweight controls could therefore mirror increased mitochondrial dysfunction in PWH. We could not assess whether either HIV or cART-use relates to CH mutation development, as all PWH were on cART and with plasma HIV RNA levels <200 copies/ml. However, our data indicate that the ROS-signature may contribute to mutations in PWH with prior exposure to zidovudine, whereas it was absent in unexposed individuals. Zidovudine, an NRTI, has been extensively linked to hematological toxicity[28].

The fact that our data suggest that in PWH other processes significantly reduce the correlation of CH prevalence with age is intriguing. Associations between clonal hematopoiesis and cART duration as well as with age and coronary artery disease were

recently reported[11]. Yet, no correlations with other HIV-related parameters such as CD4 T-cell count, HIV medication, HIV reservoir parameters were reported. We identified various other HIV-related parameters correlated with CH independently of age, such as CD4 nadir and CD4/CD8 T cell ratio. CD4 nadir is a marker for the severity of prior immunosuppression and HIV infection course. The link with CD4 nadir suggests prior immunosuppression due to HIV infection is associated with an increased CH prevalence in PWH. Recent studies have also linked CH to chronic infection and infection risk[28,29]. Longitudinal studies are needed to further investigate the link between HIV infection and CH. While in HIV-uninfected controls a link between clonal hematopoiesis and inflammation has been shown[3,30], no association was found between clonal hematopoiesis and general inflammation parameters in our cohort of PWH. Our sample size restricted our ability to dissect whether inflammation independent of CD4 nadir has an effect. In contrast, new factors such as HIV reservoir and markers of coagulation – d-dimer and vWF - were independently associated with clonal hematopoiesis. Besides inflammatory markers, these coagulation factors have also been linked to mortality and CVD and are increased in PWH[27,31]. The interplay between coagulation, inflammation and CH requires future mechanistic studies.

The link with HIV reservoir is interesting, considering that several of the most important clonal hematopoiesis driver genes encode for epigenetic modifiers - genes that modify the epigenome through *e.g.* DNA methylation - with suggested downstream effects of altered gene expression and increased inflammation[30]. HIV transcription is known to rely on epigenetic modification, cell differentiation and activation but also low-grade inflammation[1]. It is intriguing to speculate, that increased relative HIV-1 transcription in individuals with CH could be the result of an aberrant epigenetic profile, causing alterations in the transcriptional program and cell differentiation[15]. Future studies using single cell transcriptome analyses in a longitudinal setting, could provide mechanistical insight into clonal hematopoiesis and HIV reservoir dynamics on single cell level. These studies could elucidate new mechanisms for (deep) HIV latency and influence future shock and kill strategies in HIV cure [32].

Limitations of the current study include the chosen control populations. The 300-Obese control individuals were processed with the exact same assay and in parallel with PWH, preventing possible confounding due to assay or batch differences, but they were generally older and showed an increased BMI. Although at the same time, the inclusion of these controls with additional cardiovascular risk factors and older age could underestimate the effect of HIV infection itself on clonal hematopoiesis, since both age and obesity has been found associated to increased prevalence of CH[16]. The comparisons between PWH individuals and population controls are more appropriate due to age- and sex-matching, but the assay is slightly different and as such we could only validate findings for non-*DNMT3A* mutations. Additionally, our limited sample size restricted the ability to link CH to non-AIDS related comorbidities, further examine signature analyses or perform sex-specific analyses. Furthermore, although the reported associations are interesting, the data do not allow us to draw causal inferences. The purpose of this study was exploratory and as such the reported independent correlations should be interpreted as hypothesis generating and require confirmation in future, ideally longitudinally designed, studies. And finally, our targeted sequencing technique limits unbiased genome wide CH mutation assessment[3], due to only targeting previously described CH mutation hotspots and genes. Although, this is outweighed by the advantages of this technique - the use of high-sensitivity targeted sequencing including molecular barcoding allows for technical replication, comparable sensitivity, reliable quantification and overlapping probes ruling out sequencing errors or artefacts[15].

In conclusion, clonal hematopoiesis in PWH using long-term cART is associated with low CD4 nadir, increased CD4/CD8 T-cell ratio, altered coagulation and signs of increased HIV transcriptional activity. Future studies on the effect of clonal hematopoiesis on the HIV reservoir, and non-AIDS related comorbidities are warranted.

Supplementary material

All supplementary figures, the supplementary methods, and supplementary tables 1A, 2-7 can be found at the end of this manuscript. Supplementary table 2 is available online or upon request in a separate excel file.

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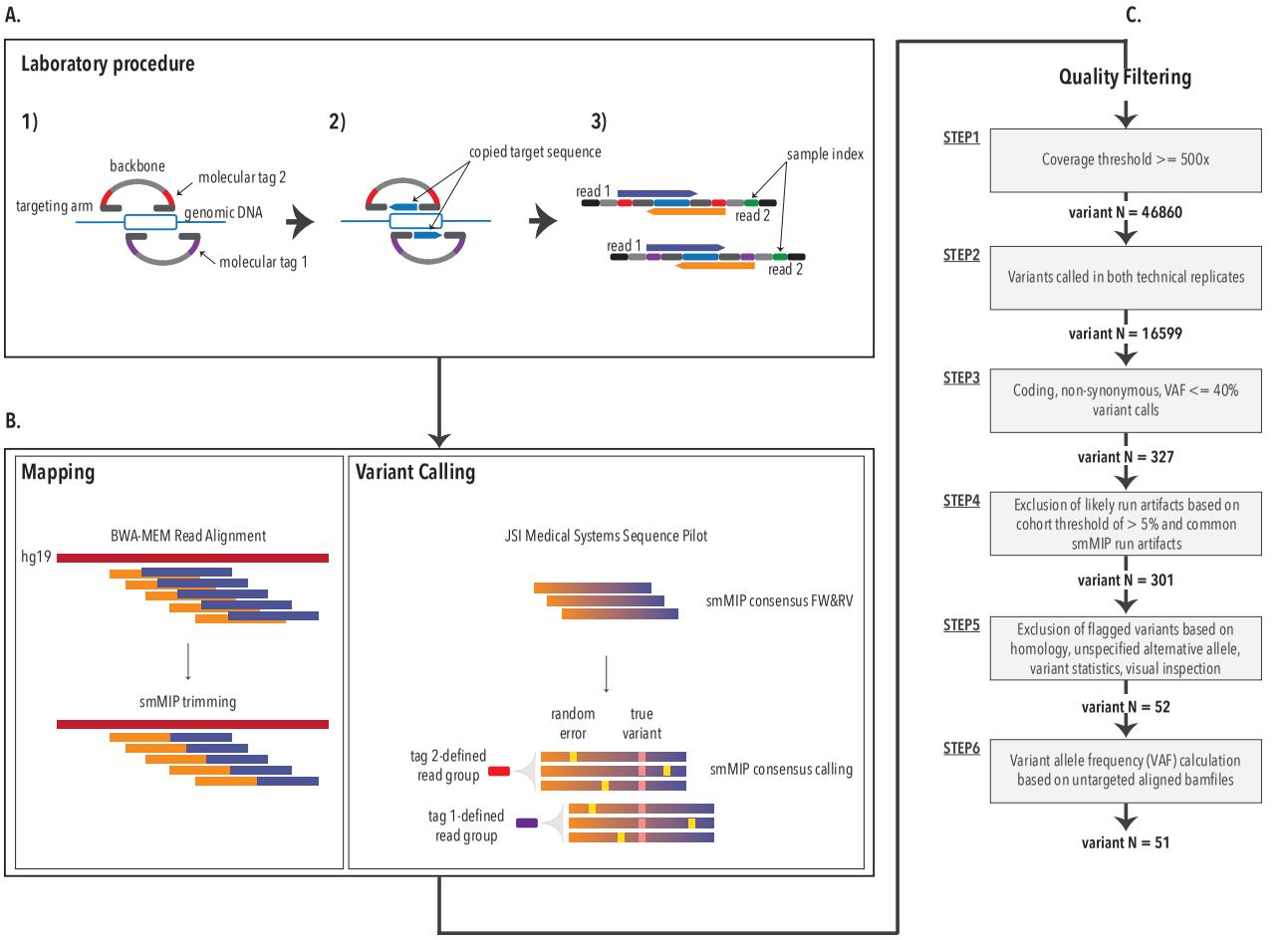
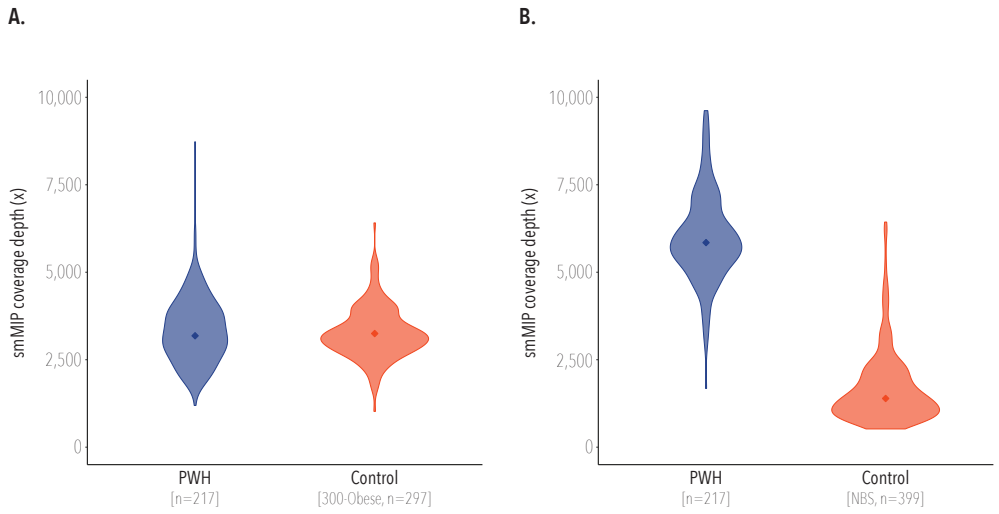
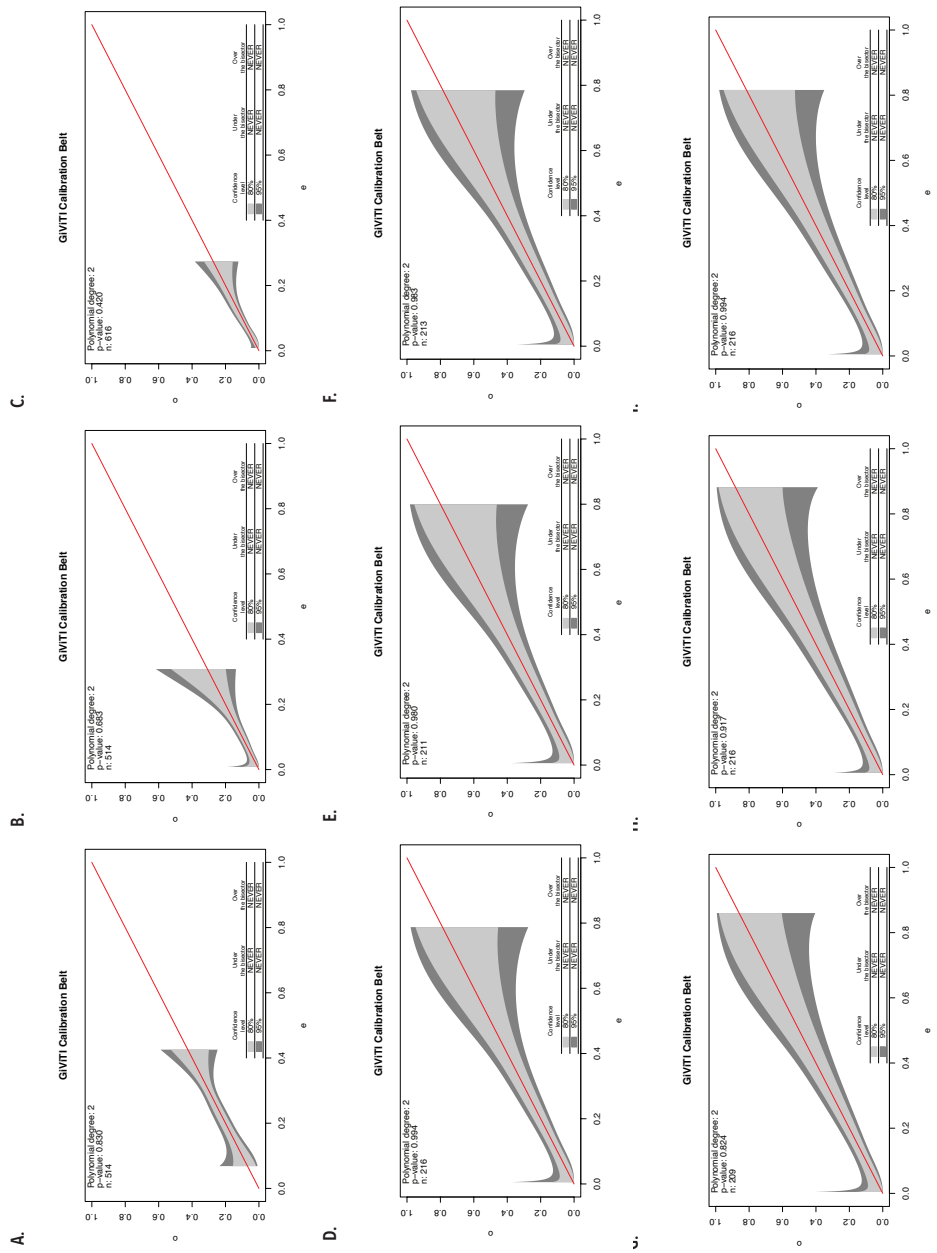


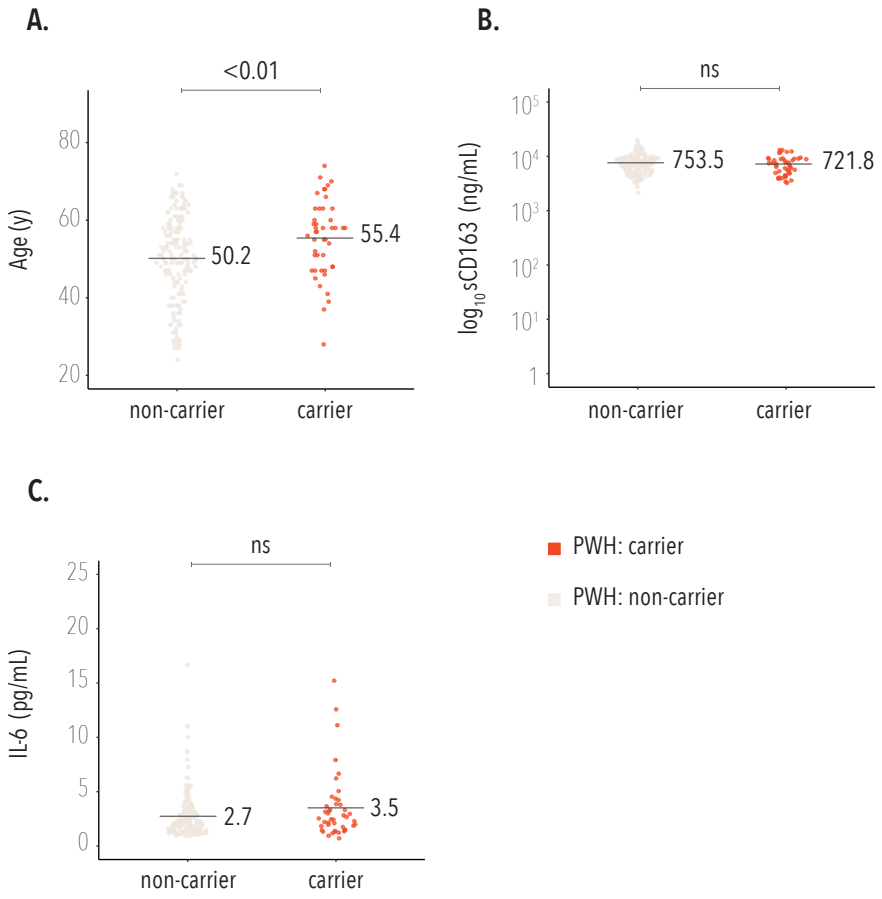
Supplementary Figure S1. smMIP workflow - adapted from Hiatt et al. 2013. to further boost sensitivity for low-level somatic mutation detection. (A) smMIP insert-sizes were shortened to 54nt to enable full forward and reverse read coverage (double sequencing of each insert), target-sequences were generally targeted by at least two independent smMIP-probes (double tiling), and each smMIP capture underwent two independently barcoded PCR reactions (double PCR replicates). Sequencing was performed using the Illumina NextSeq 500 system. (B) Raw sequencing data was converted to fastQ-files after which two independent data processing strategies were applied. Mapping: FastQ-file reads were aligned to the reference genome (Hg19) with BWA-MEM after which the overlap between Forward (FW) and Reverse (RV) reads was trimmed off (smMIP trimming). Variant Calling: FastQ-files were imported in JSI Medical Systems Sequence Pilot in which first a consensus between FW and RV reads is determined (smMIP consensus FW&RV), after which tag defined read groups enable smMIP consensus calling. (C) The aligned reads and resulting variant calls are then subjected to a stringent quality filtering pipeline. First, individuals with an average sequencing depth < 500x are excluded (STEP1). Second, only variants called in both technical replicates are kept (STEP2). Third, coding, non-synonymous variant calls with a Variant Allele Frequency (VAF) <40% are kept (STEP3). Fourth, likely run specific artefacts (variant calls in >5% of samples per run) are excluded (STEP4). Fifth, exclusion of variants based on homology, unspecified alternative allele, variant statistics and visual inspection (STEP5). And finally, for each variant position we generated mpileups based on the aligned reads to determine the final VAF for each CHDM.



Supplementary Figure S3. smMIP coverage PLHIV and controls. (**A**) shows the average coverage depth for PLHIV (n=217) and HIV-uninfected controls in red (300-Obese, n-297) over the entire Clonal hematopoeisis panel, the blue and red diamonds represent the mean coverage depth for all individuals of the respective cohort. (**B**) shows the average coverage depth for PLHIV (n=217) and HIV-uninfected population controls (n=399) over all non-DNMT3A genes in the Clonal hematopoeisis panel, the blue and red diamonds represent the mean coverage depth for all individuals of the respective cohort.



Supplementary Figure S2. GiViTI calibration belt plots for Logistic Models in Supplementary Table S7. (**A**) GiViTI calibration belt plot for Logistic Model I. (**B**) GiViTI calibration belt plot for Logistic Model II. (**C**) GiViTI calibration belt plot for Logistic Model III. (**D**) GiViTI calibration belt plot for Logistic Model IV. (**E**) GiViTI calibration belt plot for Logistic Model V. (**F**) GiViTI calibration belt plot for Logistic Model VI. (**G**) GiViTI calibration belt plot for Logistic Model VII. (**H**) GiViTI calibration belt plot for Logistic Model VIII. (**I**) GiViTI calibration belt plot for Logistic Model VIII.



Supplementary Figure S4. (A) Clonal hematopoeisis mutation carriers (n=46) are significantly older as compared to non-carriers (n=171). (B) CHDM-carriers (n=45) present with comparable soluble CD163 (sCD163) levels as compared to non-carriers (n=170). (C) CHDM-carriers (n=44) show non-significantly increased interleukin (IL)-6 as compared to non-carriers (n=168). In each plot the mean of non-carriers and CHDM-carriers is annotated with a horizontal bar and the mean to the right, at the top the Wilcoxon-rank sum test P-values is depicted.

Supplementary Table S1. Gene regions sequenced

Gene	CHR	BPstart	BPstop	Hotspot(s)
GNB1	chr1	1747219	1747281	K57
NRAS	chr1	115256481	115256578	Q61
NRAS	chr1	115258695	115258772	G13
DNMT3A	chr2	25457097	25457337	R882; F868
DNMT3A	chr2	25458534	25458737	W860
DNMT3A	chr2	25459766	25459898	
DNMT3A	chr2	25461963	25462122	P777
DNMT3A	chr2	25463116	25463351	R771; G762; Y735
DNMT3A	chr2	25463462	25463661	
DNMT3A	chr2	25464379	25464623	
DNMT3A	chr2	25466728	25466891	
DNMT3A	chr2	25466975	25467236	
DNMT3A	chr2	25467371	25467573	
DNMT3A	chr2	25468094	25468246	
DNMT3A	chr2	25468860	25468966	
DNMT3A	chr2	25468999	25469230	
DNMT3A	chr2	25469451	25469670	
DNMT3A	chr2	25469890	25470072	
DNMT3A	chr2	25470425	25470644	R326
DNMT3A	chr2	25470847	25471147	
DNMT3A	chr2	25497766	25497976	
DNMT3A	chr2	25498336	25498443	
DNMT3A	chr2	25505264	25505647	
DNMT3A	chr2	25522970	25523163	
DNMT3A	chr2	25536724	25536884	
SF3B1	chr2	198266793	198266879	K700
SF3B1	chr2	198267310	198267375	K666
MYD88	chr3	38182285	38182347	L265
MYD88	chr3	38182614	38182677	L273
PIK3CA	chr3	178916710	178916775	R38
FGFR3	chr4	1803523	1803588	S249
FGFR3	chr4	1806082	1806145	G380
FGFR3	chr4	1807841	1807923	K650
TET2	chr4	106156676	106156762	L532; R550
TET2	chr4	106157359	106157422	Q764
TET2	chr4	106157586	106157655	H839

TET2	chr4	106157712	106157776	Q888
BRAF	chr7	140453103	140453168	V600
BRAF	chr7	140481373	140481439	G469
BRAF	chr7	140494133	140494201	R362
JAK2	chr9	5073728	5073801	V617
PTEN	chr10	89692869	89692955	R130
FGFR2	chr10	123279627	123279726	P253
HRAS	chr11	534241	534335	G12
CBL	chr11	119148879	119149040	C381; C396; C404
CBL	chr11	119149194	119149295	F418
KRAS	chr12	25398233	25398313	G12
PTPN11	chr12	112888146	112888211	Y63
IDH2	chr15	90631915	90631981	R140
TP53	chr17	7577460	7577612	I255; G187; R248
TP53	chr17	7578157	7578226	Y220
TP53	chr17	7578374	7578461	R175
TP53	chr17	7579327	7579393	R110
STAT3	chr17	40474385	40474451	D661
SRSF2	chr17	74732933	74733004	P95
ASXL1	chr20	31021185	31021291	R404; R417
ASXL1	chr20	31022246	31022314	Y591
ASXL1	chr20	31022573	31022637	Y693
ASXL1	chr20	31022891	31022956	Q803
GNAS	chr20	57484390	57484467	R844
U2AF1	chr21	44524436	44524500	S34
BRCC3	chrX	154305462	154305526	R81

Supplementary Table S3.

Probes and Primers for HIV-1 cell-associated RNA and DNA

Assay	Oligo	Modification	Sequence
cell-associated HIV-1 DNA and cell-associated HIV-1 RNA	Forward		5'-GCCTCAATAAAGCTTGCC-3'
	Reverse		5'-GGCGCCACTGCTAGAGATTTT-3'
	Probe	6FAM/MGB Eclipse	5'-AAGTRGTGTGTGCCC-3'
Reference gene RPP30	Forward		5'-AGATTTGGACCTGCGAGCG-3'
	Reverse		5'-GAGCGGCTGTCTCCACAAGT-3'
	Probe	HEX/ZEN/3' IBFQ	5'-TTCTGACCTGAAGGCTCTGCGCG-3'
Reference gene B2M	Forward		5'-TGTCGGATGGATGAAACCCAGA-3'
	Reverse		5'-TGCTCGCGCTACTCTCTCTTT-3'
Reference gene ACTB	Forward		5'-CCGATCCACACGGAGTACTT-3'
	Reverse		5'-TGGACATCCGCAAAGACCTG-3'
Reference gene GAPDH	Forward		5'-GAAGATGGTGATGGGATTTC-3'
	Reverse		5'-GAAGGTGAAGGTCGGAGTC-3'

Supplementary Table S4.

SubjectID	JSIgene	JSIgPos	JSIcHGVS	JSIpHGVS	JSIaaChange	JSInucChange	mpileupVAF
HIV2	SF3B1	chr2:g.198267360	c.1997A>C	p.Lys666Thr	K -> T (666)	A_->_C_(het)	1.67
HIV4	CBL	chr11:g.119149251	c.1259G>A	p.Arg420Gln	R -> Q (420)	G_->_A_(het)	0.13
HIV5	DNMT3A	chr2:g.25463286	c.2207G>A	p.Arg736His	R -> H (736)	G_->_A_(het)	7.79
HIV8	GNAS	chr20:g.57484421	c.2531G>A	p.Arg844His	R -> H (844)	G_->_A_(het)	0.05
HIV10	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.28
HIV12	CBL	chr11:g.119149250	c.1258C>T	p.Arg420Ter	R -> [STOP] (420)	C_->_T_(het)	0.05
HIV22	DNMT3A	chr2:g.25471070	c.691C>T	p.Gln231Ter	Q -> [STOP] (231)	C_->_T_(het)	10.96
HIV25	DNMT3A	chr2:g.25463184	c.2309C>T	p.Ser770Leu	S -> L (770)	C_->_T_(het)	2.09
HIV33	BRAF	chr7:g.140481403	c.1405G>C	p.Gly469Arg	G -> R (469)	G_->_C_(het)	0.53
HIV42	TET2	chr4:g.106156729	c.1693C>T	p.Arg565Ter	R -> [STOP] (565)	C_->_T_(het)	0.07
HIV45	DNMT3A	chr2:g.25469945	c.1097G>A	p.Arg366His	R -> H (366)	G_->_A_(het)	17.32
HIV50	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.48
HIV66	DNMT3A	chr2:g.25467479	c.1597T>C	p.Tyr533His	Y -> H (533)	T_->_C_(het)	2.92
HIV66	DNMT3A	chr2:g.25463286	c.2207G>A	p.Arg736His	R -> H (736)	G_->_A_(het)	1.19
HIV82	DNMT3A	chr2:g.25457176	c.2711C>T	p.Pro904Leu	P -> L (904)	C_->_T_(het)	0.35
HIV88	STAT3	chr17:g.40474420	c.1981G>T	p.Asp661Tyr	D -> Y (661)	G_->_T_(het)	0.32
HIV90	DNMT3A	chr2:g.25457158	c.2729C>T	p.Ala910Val	A -> V (910)	C_->_T_(het)	0.53
HIV91	JAK2	chr9:g.5073770	c.1849G>T	p.Val617Phe	V -> F (617)	G_->_T_(het)	1.13
HIV94	STAT3	chr17:g.40474420	c.1981G>T	p.Asp661Tyr	D -> Y (661)	G_->_T_(het)	0.21
HIV95	STAT3	chr17:g.40474420	c.1981G>T	p.Asp661Tyr	D -> Y (661)	G_->_T_(het)	0.78
HIV101	DNMT3A	chr2:g.25463289	c.2204A>G	p.Tyr735Cys	Y -> C (735)	A_->_G_(het)	2.09
HIV108	DNMT3A	chr2:g.25457176	c.2711C>A	p.Pro904Gln	P -> Q (904)	C_->_A_(het)	1.32
HIV113	DNMT3A	chr2:g.25463578	c.2104G>A	p.Asp702Asn	D -> N (702)	G_->_A_(het)	7.2
HIV116	DNMT3A	chr2:g.25462068	c.2339T>C	p.Ile780Thr	I -> T (780)	T_->_C_(het)	0.35
HIV131	DNMT3A	chr2:g.25463308	c.2185C>T	p.Arg729Trp	R -> W (729)	C_->_T_(het)	1.16
HIV137	DNMT3A	chr2:g.25463182	c.2311C>T	p.Arg771Ter	R -> [STOP] (771)	C_->_T_(het)	0.34
HIV139	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.27
HIV140	STAT3	chr17:g.40474420	c.1981G>T	p.Asp661Tyr	D -> Y (661)	G_->_T_(het)	0.43
HIV141	SRSF2	chr17:g.74732959	c.284C>A	p.Pro95His	P -> H (95)	C_->_A_(het)	16.33
HIV142	CBL	chr11:g.119149251	c.1259G>A	p.Arg420Gln	R -> Q (420)	G_->_A_(het)	0.15
HIV142	GNAS	chr20:g.57484420	c.2530C>A	p.Arg844Ser	R -> S (844)	C_->_A_(het)	0.22
HIV147	DNMT3A	chr2:g.25457242	c.2645G>A	p.Arg882His	R -> H (882)	G_->_A_(het)	32.94
HIV150	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.14
HIV157	DNMT3A	chr2:g.25463287	c.2206C>T	p.Arg736Cys	R -> C (736)	C_->_T_(het)	4.39
HIV162	DNMT3A	chr2:g.25467188	c.1687G>A	p.Val563Met	V -> M (563)	G_->_A_(het)	14.42
HIV164	DNMT3A	chr2:g.25458663	c.2510C>A	p.Ser837Ter	S -> [STOP] (837)	C_->_A_(het)	3.61
HIV169	DNMT3A	chr2:g.25466802	c.1901T>G	p.Ile634Ser	I -> S (634)	T_->_G_(het)	3.13
HIV169	DNMT3A	chr2:g.25457242	c.2645G>A	p.Arg882His	R -> H (882)	G_->_A_(het)	0.69
HIV175	GNB1	chr1:g.1747229	c.169A>G	p.Lys57Glu	K -> E (57)	A_->_G_(het)	4.73
HIV175	TP53	chr17:g.7577547	c.734G>C	p.Gly245Ala	G -> A (245)	G_->_C_(het)	6.05
HIV183	DNMT3A	chr2:g.25457176	c.2711C>T	p.Pro904Leu	P -> L (904)	C_->_T_(het)	0.89
HIV189	DNMT3A	chr2:g.25457242	c.2645G>A	p.Arg882His	R -> H (882)	G_->_A_(het)	6.57
HIV201	DNMT3A	chr2:g.25470516	c.958C>T	p.Arg320Ter	R -> [STOP] (320)	C_->_T_(het)	6.21
HIV212	DNMT3A	chr2:g.25463587	c.2095G>A	p.Gly699Ser	G -> S (699)	G_->_A_(het)	11.54
HIV213	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.63
HIV213	DNMT3A	chr2:g.25463181	c.2312G>A	p.Arg771Gln	R -> Q (771)	G_->_A_(het)	0.37
HIV215	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.35
HIV218	JAK2	chr9:g.5073728	c.1807A>G	p.Lys603Glu	K -> E (603)	A_->_G_(het)	0.79

(A) All detected mutations in PLHIV and all detected single base substitution mutations in PLHIV.

Supplementary Table S6. Baseline characteristics of PLHIV vs controls.

Parameter	PLHIV (n=217)	Control (n=297)	P-value
Sex Female - n (%)	18 (8.3%)	163 (54.9%)	<0.01
Age - mean [IQR] (years)	51.3 [46-59]	67.1 [63-71]	<0.01
BMI - mean [IQR] (kg/m2)	24.5 [22.1-26.2]	30.7 [28.3-31.9]	<0.01
Smoking - n (%)	61 (28.1%)	29 (9.8%)	<0.01
CHDM - n	51	110	na
CHDM carriers - n (%)	46 (21.1%)	85 (28.6%)	0.07
CHIP - n	20	37	na
CHIP carriers - n (%)	19 (6.4%)	33 (11.1%)	0.47

Thecolumn P- value represents the X-square test P-value for categorical variables, and the Wilcoxon- rank-sumtest P-valueinthecaseof continuousvariables.IQR=Interquartilerange.

Supplementary Table S7. Logistic regression models

Logistic Model I :: CH mutation yes/no ~ age + cohort [n=514]

	Estimate	p-value	OR	Lower	Upper
(Intercept)	-3,952	2,78E-05	0,019	0,003	0,122
cohort - PLHIV	0,259	0,359	1,296	0,745	2,253
age	0,045	0,001	1,046	1,018	1,075

Logistic Model II :: CHIP yes/no ~ age + cohort [n=514]

	Estimate	p-value	OR	Lower	Upper
(Intercept)	-7,511	5,67E-07	0,001	2,88E-05	0,010
cohort - PLHIV	0,793	0,043	2,211	1,025	4,769
age	0,080	2,21E-04	1,083	1,038	1,130

Logistic Model III :: CH mutation yes/no ~ age + cohort [n=616]

	Estimate	p-value	OR	Lower	Upper
(Intercept)	-7,511	5,67E-07	0,001	2,88E-05	0,010
cohort - PLHIV	0,793	0,043	2,211	1,025	4,769
age	0,080	2,21E-04	1,083	1,038	1,130

Logistic Model IV :: PLHIV CH mutation yes/no~ age + CD4 nadir + CD4/CD8 ratio [n=216]

	Estimate	p-value	OR	Lower	Upper	Increment
(Intercept)	-3,175	4,04E-03				
age	0,037	4,09E-02	1,205	1,013	1,450	5
CD4 nadir	-0,005	0,001	0,794	0,687	0,907	50
CD4/CD8 ratio	1,047	0,008	2,850	1,334	6,391	1

Logistic Model V :: PLHIV CH mutation yes/no~ age + CD4 nadir + CD4/CD8 ratio + log(DNA) [n=211]

	Estimate	p-value	OR	Lower	Upper	Increment
(Intercept)	-3,062	0,077				
age	0,043	0,023	1,240	1,036	1,503	5
CD4 nadir	-0,005	2,09E-03	0,796	0,684	0,916	50
CD4/CD8 ratio	1,044	9,89E-03	2,842	1,308	6,487	1
log(DNA)	-0,132	0,754	0,876	0,385	2,033	

Logistic Model VI :: PLHIV CH mutation yes/no~ age + CD4 nadir + CD4/CD8 ratio + log(RNA) [n=213]

	Estimate	p-value	OR	Lower	Upper	Increment
(Intercept)	-4,567	3,63E-03				
age	0,041	0,031	1,225	1,025	1,483	5
CD4 nadir	-0,004	0,011	0,824	0,705	0,954	50
CD4/CD8 ratio	1,077	0,007	2,937	1,364	6,645	1
log(RNA)	0,462	0,308	1,588	0,661	3,949	

Logistic Model VII :: PLHIV CH mutation yes/no~ age + CD4 nadir + CD4/CD8 ratio + RNA/DNA ratio [n=209]

	Estimate	p-value	OR	Lower	Upper	Increment
(Intercept)	-3,575	1,82E-03				
age	0,040	3,40E-02	1,221	1,021	1,480	5
CD4 nadir	-0,005	1,55E-03	0,795	0,684	0,911	50
CD4/CD8 ratio	1,008	0,012	2,740	1,265	6,210	1
RNA/DNA ratio	1,716	0,017	5,563	1,768	45,970	

Logistic Model VIII :: PLHIV CH mutation yes/no~ age + CD4 nadir + CD4/CD8 ratio + vWF [n=216]

	Estimate	p-value	OR	Lower	Upper	Increment
(Intercept)	-3,636	1,55E-03				
age	0,032	8,40E-02	1,176	0,983	1,424	5
CD4 nadir	-0,005	1,33E-03	0,794	0,685	0,909	50
CD4/CD8 ratio	1,179	0,004	3,251	1,501	7,440	1
vWF	1,03E-05	0,009	1,053	1,014	1,097	5000

Logistic Model IX :: PLHIV CH mutation yes/no~ age + CD4 nadir + CD4/CD8 ratio + d-dimer [n=216]

	Estimate	p-value	OR	Lower	Upper	Increment
(Intercept)	-3,099	4,96E-03				
age	0,027	1,47E-01	1,146	0,957	1,387	5
CD4 nadir	-0,005	9,09E-04	0,790	0,683	0,903	50
CD4/CD8 ratio	1,077	0,007	2,937	1,364	6,647	1
D-dimer	0,001	0,044	1,076	1,004	1,163	50

Logistic regression model of clonal hematopoeisis (CH) mutation prevalence as predicted by age in PLHIV as compared to HIV-uninfected overweight controls (Logistic Model I). CHIP (clones with VAF >2%) prevalence as predicted by age in PLHIV as compared to HIV-uninfected overweight controls (Logistic Model II). Logistic regression model of non-DNMT3A mutation prevalence as predicted by age in PLHIV as compared to HIV-uninfected population controls (Logistic Model III). Logistic regression model of CHDM prevalence as predicted by age, CD4 nadir (cells/uL) and CD4/CD8 ratio (Logistic Model IV). This model was determined after a backward elimination model including multiple clinical parameters (Body mass index, known duration of HIV infection, current CD4 count, HIV RNA zenith, treatment duration, smoking (packyears), high sensitive C-reactive protein). Logistic regression model including parameters from model IV with log(DNA) (Logistic Model V). Logistic regression model including parameters from model III with log(RNA) (Logistic Model VI). Logistic regression model including parameters from model III with RNA/DNA ratio (Logistic Model VII). Logistic regression model including parameters from model III with von willebrand factor (vWF) (Logistic Model VIII). Logistic regression model including parameters from model III with d-dimer concentrations (Logistic Model IX).

Supplementary TableS6.Mutational SignatureAnalysis Results.

(A)			(B)		
Signature	**Sample**	**Contribution**	**Signature**	**Sample**	**Contribution**
SBS1	PLHIV	0,146839651	SBS1	PLHIV_noAZT	0
SBS2	PLHIV	0	SBS2	PLHIV_noAZT	0
SBS3	PLHIV	0	SBS3	PLHIV_noAZT	0
SBS4	PLHIV	0	SBS4	PLHIV_noAZT	0
SBS5	PLHIV	0	SBS5	PLHIV_noAZT	1,52E-08
SBS6	PLHIV	0	SBS6	PLHIV_noAZT	0,229738216
SBS7a	PLHIV	0	SBS7a	PLHIV_noAZT	0
SBS7b	PLHIV	0	SBS7b	PLHIV_noAZT	0
SBS7c	PLHIV	0	SBS7c	PLHIV_noAZT	0
SBS7d	PLHIV	0	SBS7d	PLHIV_noAZT	0
SBS8	PLHIV	0	SBS8	PLHIV_noAZT	0
SBS9	PLHIV	0	SBS9	PLHIV_noAZT	0
SBS10a	PLHIV	0	SBS10a	PLHIV_noAZT	0
SBS10b	PLHIV	0,099071028	SBS10b	PLHIV_noAZT	0,093191123
SBS11	PLHIV	0	SBS11	PLHIV_noAZT	0
SBS12	PLHIV	0,071913016	SBS12	PLHIV_noAZT	0,096705966
SBS13	PLHIV	0	SBS13	PLHIV_noAZT	0
SBS14	PLHIV	0	SBS14	PLHIV_noAZT	0
SBS15	PLHIV	0	SBS15	PLHIV_noAZT	0
SBS16	PLHIV	0	SBS16	PLHIV_noAZT	0
SBS17a	PLHIV	0	SBS17a	PLHIV_noAZT	0
SBS17b	PLHIV	0	SBS17b	PLHIV_noAZT	0
SBS18	PLHIV	0,157476747	SBS18	PLHIV_noAZT	0
SBS19	PLHIV	0	SBS19	PLHIV_noAZT	0
SBS20	PLHIV	0	SBS20	PLHIV_noAZT	0
SBS21	PLHIV	0	SBS21	PLHIV_noAZT	0
SBS22	PLHIV	0	SBS22	PLHIV_noAZT	0
SBS23	PLHIV	0	SBS23	PLHIV_noAZT	0
SBS24	PLHIV	0	SBS24	PLHIV_noAZT	0
SBS25	PLHIV	0	SBS25	PLHIV_noAZT	0
SBS26	PLHIV	0	SBS26	PLHIV_noAZT	0
SBS28	PLHIV	0	SBS28	PLHIV_noAZT	0
SBS29	PLHIV	0	SBS29	PLHIV_noAZT	0,07230757
SBS30	PLHIV	0,038096552	SBS30	PLHIV_noAZT	0,052809567
SBS31	PLHIV	0,486603007	SBS31	PLHIV_noAZT	0,455247543
SBS32	PLHIV	0	SBS32	PLHIV_noAZT	0
SBS33	PLHIV	0	SBS33	PLHIV_noAZT	0
SBS34	PLHIV	0	SBS34	PLHIV_noAZT	0
SBS35	PLHIV	0	SBS35	PLHIV_noAZT	0
SBS36	PLHIV	0	SBS36	PLHIV_noAZT	0
SBS37	PLHIV	0	SBS37	PLHIV_noAZT	0
SBS38	PLHIV	0	SBS38	PLHIV_noAZT	0
SBS39	PLHIV	0	SBS39	PLHIV_noAZT	0
SBS40	PLHIV	0	SBS40	PLHIV_noAZT	0
SBS41	PLHIV	0			
SBS42	PLHIV	0			
SBS44	PLHIV	0			
SBS84	PLHIV	0			
SBS85	PLHIV	0			
SBS1	Control	0			
SBS2	Control	0			
SBS3	Control	0			

(A) PLHIV versus Control. (B) PLHIV with history of Zidovudine use (PLHIV_yesAZT) versus PLHIV without history of Zidovudine use (PLHIV_noAZT).

Chapter 5

Plasmatic coagulation capacity correlates with inflammation and abacavir-use during chronic HIV-infection

Wouter van der Heijden, Jun Wan, Lisa Van de Wijer, Martin Jaeger, Mihai Netea, Andre van der Ven, Philip de Groot, Mark Roest, Quirijn de Mast

Journal of acquired immune deficiency syndromes 2021; 87(1): 711-9.

Abstract

D-dimer concentrations in people living with HIV (PLHIV) on antiretroviral therapy (cART) are increased and have been linked to mortality. D-dimer is a biomarker of *in vivo* coagulation, In contrast to reports on D-dimer, data on coagulation capacity in PLHIV are conflicting. Here, we assessed the effect of cART and inflammation on coagulation capacity. We explored coagulation capacity using *calibrated* thrombin generation (TG) and linked this to persistent inflammation and cART in a cross-sectional study including PLHIV with viral suppression and uninfected controls. We used multivariate analyses to identify independent factors influencing *in vivo* coagulation (D-dimer) and *ex vivo* coagulation capacity (TG). Among 208 PLHIV, 94 (45%) were on an abacavir containing regimen. D-dimer levels (219.1 vs 170.5 ng/mL, p=0.001) and inflammatory makers (sCD14, sCD163 and hsCRP) were increased in PLHIV compared to controls (n=56). PLHIV experienced lower thrombin generation (reflected by endogenous thrombin potential; ETP) compared to controls, after correction for age, sex and antiretroviral therapy. Abacavir-use was independently associated with increased ETP. Prothrombin concentrations were strongly associated with ETP and were lower in PLHIV on a non-abacavir containing regimen compared to controls, suggesting consumption as a possible mechanism for HIV-associated reduction in TG. D-dimer concentrations were associated with inflammation, but not TG. Abacavir-use was associated with increased TG and could serve as an additional factor in the reported increase in thrombotic events during abacavir use. Increased exposure to triggers that propagate coagulation, such as inflammation, likely underlie increased D-dimer concentrations found in most PLHIV.

Introduction

Successful combination antiretroviral treatment (cART) has nearly normalized life expectancy of people living with HIV (PLHIV), although treatment is not able to fully reverse immune activation and persistent inflammation[1-3]. This inflammation may increase the risk for thrombotic events [4]. While increased risk for venous thrombosis PLHIV appears to be limited to those with incomplete CD4 recovery or with continuing viral replication [5,6], data on arterial thrombosis risk show an increased cardiovascular risk in well-treated PLHIV [7-9]. There is an ongoing debate on the effect of cART, especially abacavir, on cardiovascular risk [10-15].

D-dimers, which are soluble fibrin degradation products, are a marker of coagulation activity in the body. HIV infection is associated with elevated D-dimer concentrations even in those with viral suppression [16-18]. D-dimer concentrations are independently associated with overall mortality and the incidence of cardiovascular disease and cancer in PLHIV [9,16,19]. D-dimer concentrations are influenced by activation of the coagulation cascade itself, as well as signals that provoke coagulation such as inflammation and endothelial activation [4]. Indeed, the inflammatory markers sCD14, high-sensitive C-reactive protein (hsCRP) and sCD163 have been associated with D-dimer in PLHIV [17,20], suggesting a link between inflammation and *in vivo* coagulation activity in PLHIV.

The capacity of plasma to form thrombin is a critical determinant of in vivo plasmatic coagulation [21]. Thrombin generation (TG) can be measured *ex vivo* to determine coagulation capacity in a standardized setting and has been used as a diagnostic tool for hypo- and hypercoagulability states [22]. In contrast to elevated D-dimer concentrations in PLHIV [16-18], available data on *ex vivo* TG in PLHIV are contradictory [23-25]. This also applies to the possible roles of inflammation and cART on TG.

Hence, we measured TG, as well as different coagulation markers, in a cross-sectional cohort of cART treated, virally suppressed PLHIV and HIV-uninfected controls and related TG to markers of inflammation and cART. We hypothesized that TG is influenced by both persistent inflammation and cART.

Materials and Methods

Study procedures

This cross-sectional, prospective study was performed at the Radboud university medical center, a tertiary teaching hospital in The Netherlands. The study was conducted in accordance with the Declaration of Helsinki after approval of the ethics committee (CMO Arnhem-Nijmegen, The Netherlands; NL42561.091.12, 2012/550). This study was embedded in the Human Functional Genomics Project (HFGP; www.humanfunctionalgenomicsproject.org). Adult HIV-1 infected individuals and controls were concurrently enrolled after a written informed consent was obtained. Inclusion criteria included suppressed viral load (<200copies/mL) after cART use for at least six months. Exclusion criteria were the use of coumarin derivates or direct anticoagulant therapy, as well as active hepatitis B or C infection, and/or signs of an infection other than HIV-1.

Plasma thrombin generation measured with and without thrombomodulin

TG in platelet poor plasma (PPP) was measured with the "MidiCAT" technique, which is a modified calibrated thrombin generation (CAT) for the measurement of samples with low plasma volume. [22,26] This technique maintains the plasma dilution ratio while requiring only half of the volume (i.e. 40 versus 80 µL per well) needed than the regular CAT technique. PPP was stored at -80°C until thawed at 37°C for 10 min before measurement. TG was triggered with 5 pM tissue factor (TF; Innovin, Seimens Healthcare Diagnostics, Marburg, Germany), 4 µM phospholipids (PL; Avanti Polar Lipids Inc., Alabaster, AL, USA) and in the presence and absence of 7 nM thrombomodulin (TM; Synapse research institute, Maastricht the Netherlands). The concentration of TM was chosen to inhibit the ETP by 50% in normal pooled plasma (NPP). TG parameters were calculated using specialized software from Thrombinoscope B.V. (Maastricht, the Netherlands). TG parameters (supplemental figure 1) including Lagtime (LT; min), time-to-peak (TTP; min), Peak (nM), endogenous thrombin potential (ETP; nM×min) and velocity index (VI; nM/min) were chosen for further analysis. The ETP, Peak and VI of tested subjects were normalized as the percentage of that of normal pooled plasma (NPP) tested without TM in the same run.[26]

The sensitivity of the TG parameters to TM reflects the function of the anticoagulant protein C pathway. The TM sensitivity ratio of ETP (ETP-TMsr) was calculated as the ratio of the ETP in the presence of TM and ETP in the absence of TM. The normalized TM sensitivity ratio of ETP (nETP-TMsr) was calculated by dividing the ETP-TMsr of subject by that of NPP in the same run. The nPeak-TMsr and nVI-TMsr were calculated similarly. A nTMsr value less than 1 means that the PPP of the tested subject have a better functioning protein C system than the NPP, and vice versa.

Plasma markers of coagulation and inflammation

Inflammatory markers sCD163 (Quantikine), sCD14 (Quantikine) and hsCRP (Duoset) were determined in EDTA plasma by ELISA (all R&D system, Minneapolis, USA). D-dimer was measured by ELISA according manufacturer's instructions (Abcam, Cambridge, UK).

The measurement of plasmatic fibrinogen was performed using the Clauss-method on a Start4 analyzer (Dignostica Stago, Asnières, France) with a known fibrinogen reagent (Dade® Fibrinogen Determination Reagents, Siemens, Munich, Germany). Prothrombin (sheep anti-human prothrombin polyclonal antibody and HRP-conjugated sheep anti-human prothrombin polyclonal antibody; Affinity Biologicals, Ancaster, Canada), protein S (sheep anti-human protein S IgG antibody and HRP-conjugated sheep anti-human protein S antibody from Affinity Biologicals and von Willebrand factor (vWF; Rabbit anti-human vWF and HRP-conjugated rabbit anti human vWF; DAKO, Agilent, Santa Clara, USA) concentrations were performed with an in-house sandwich ELISA assay. Briefly, 96 wells microtiter plates (NUNC Maxisorp, Thermo Fisher Scientific, Waltham, USA) were coated overnight at 4°C with capture antibody in a carbonate-bicarbonate coating buffer (pH 9.6) and blocked with 2% BSA in phosphate-buffered saline (PBS) for 45 minutes at room temperature (RT) before adding diluted plasma samples and incubated at RT for 1.5 hour. The wells were then incubated with in detection antibody in PBS/2%BSA for 2 hours at room temperature after washing. Plates were washed before addition of SIGMAFAST OPD (Sigma). After 30 minutes the reaction was stopped with 3 M sulfuric acid (H_2SO_4, Sigma). Optical densities (OD) were measured at 490 nm using an ELx808 Absorbance Microplate Reader (Biotek, Bad Friedrichshall, Germany). A calibration curve of serial diluted NPP was added to each plate [27,28]. Consequently, the concentrations of prothrombin and protein S were expressed as the percentage (%) of the normal NPP.

Statistical analysis

R version 3.5.1 (CRAN-project) was used for analyses. Comparison between groups was done by Mann-Whitney U test, Student's T-test or Chi Square test depending data distribution. The primary outcome for TG used in our analyses was ETP. Other TG parameters were treated as exploratory parameters. Benjamini-Hochberg procedure was performed on circulating markers and ETP comparisons (Figure 2, Table 2). For univariate and multivariate linear regression data of the dependent variable was transformed by log- or inverse rank transformation depending distribution. All multivariate linear regression models include a parameter to correct for possible storage degradation or time of inclusion bias. The correlation matrix was performed using spearman's correlation coefficient. Missing data for all parameters was below <2.5% and comparisons were done pairwise.

Results

A total of 208 virally suppressed PLHIV on stable cART and 56 uninfected controls were concurrently measured and included in the analysis. Baseline characteristics can be found in table 1. PLHIV were more often male (91.3% vs 60.7% p<0.001) and older (52 [46-59] vs 30 [26-53] years p<0.001) compared to HIV uninfected controls. An abacavir containing regimen was used by 94 PLHIV (45%) and 140 PLHIV (67%) used an integrase inhibitor (INSTI)-based regimen.

First, *in vivo* coagulation activity, determined by D-dimer, was increased in PLHIV compared to controls (219.1 vs 170.5 ng/mL respectively p=0.001; table 2), as were all markers of inflammation (hsCRP, sCD14, sCD163) and the microbial translocation marker IFABP (data shown in table 2). D-dimer was independently associated with age (B= 7.44, p<0.001) and after correction for age and sex, the difference in D-dimer between PLHIV and uninfected controls disappeared (B= 0.154, p=0.878;supplemental table 1). The markers of inflammation and microbial translocation remained significantly increased in PLHIV compared to controls after correction for age and sex (supplemental table 1). D-dimer concentrations correlated with markers of inflammation, IFABP, and endothelial activation (plasma vWF; see *Figure 1*).

Table 1. Baseline characteristics

Characteristics	no ABC (n=114)	ABC (n=94)	Healthy controls (n=56)	ABC vs no-ABC	HC vs PLHIV
Sex (Female) (%)	13 (11.4)	5 (5.3)	22 (39.3)	0.192	<0.001
Age (years)	53.0 [47.0, 60.0]	50.0 [41.5, 58.0]	30.0 [25.8, 53.0]	0.086	<0.001
BMI (kg/m²)	24.2 [22.4, 26.0]	23.8 [21.8, 26.2]	23.8 [21.5, 25.6]	0.469	0.518
HIV infection duration (years)	10.0 [6.2, 16.8]	6.5 [4.3, 10.3]		<0.001	
Way of transmission (%)				0.245	
Heterosexual	3 (2.6)	5 (5.3)			
IDU	2 (1.8)	1 (1.1)			
MSM	82 (71.9)	75 (79.8)			
Other/unknown	27 (23.7)	13 (13.8)			
CD4 nadir (cells/µL)	205.0 [120.0, 347.5]	275.0 [185.0, 377.5]		0.044	
CD4 count (cells/µL)	645.0 [482.5, 827.5]	665.0 [492.5, 800.0]		0.754	
Viral load <40 copies/mL (%)	111 (97.3)	94 (100%)		0.317	
CD4-CD8 ratio	0.8 [0.5, 1.1]	0.8 [0.6, 1.1]		0.954	
cART duration (years)	7.9 [4.9, 15.4]	5.5 [3.7, 8.6]		<0.001	
NNRT (%)	44 (38.6)	17 (18.1)		0.002	
PI (%)	26 (22.8)	6 (6.4)		0.002	
INSTI (%)	67 (58.8)	73 (77.7)		0.006	
NRTI FTC (%)	91 (79.8)	0 (0.0)		<0.001	
NRTI 3TC (%)	14 (12.3)	91 (96.8)		<0.001	
NRTI AZT (%)	3 (2.6)	1 (1.1)		0.755	
NtRTI TDF (%)	92 (80.7)	1 (1.1)		<0.001	
Prior myocardial infarction (%)	6 (5.3)	4 (4.3)		0.99	
Prior stroke (%)	2 (1.8)	1 (1.1)		1	
VTE/pulmonary embolism (%)	3 (2.6)	1 (1.1)		0.755	
Smoking (%)	33 (28.9)	24 (25.5)		0.694	
Hypercholesterolemia (%)	33 (28.9)	23 (24.5)		0.57	
Hypertension (%)	23 (20.2)	15 (16.0)		0.546	
Diabetes Mellitus (%)	6 (5.3)	2 (2.1)		0.419	
Family history of CVD 1st degree (%)	59 (51.8)	44 (46.8)		0.568	
cholesterol lowering drugs (%)	35 (30.7)	21 (22.3)		0.232	
Antihypertensive drugs (%)	27 (23.7)	20 (21.3)		0.805	
ASA (%)	10 (8.8)	8 (8.5)		1	

ABC: abacavir; IDU: intravenous drug-use; MSM: man who have sex with man; HIVRNA blib: viral load of 40-500 copies/mL after previous viral suppression and direct subsequent suppressed viral load; NRTI: nucleoside reverse transcriptase inhibitor; NtRTI: nucleotide reverse transcriptase inhibitor; NNRTI: non-nucleoside reverse transcriptase inhibitor; PI: protease inhibitor; INSTI: integrase inhibitor; FTC: emtricitabine; 3TC: lamivudine; AZT: zidovudine; TDF: Tenofovir difumarate; ASA: acetyl salicylic acid; CVD: cardiovascular disease. Data are presented as median with interquartile range and were analyzed using either chi-square test or Mann-Whitney U-test depending data distribution.

Table 2. Markers of inflammation and thrombin generation.

	PLHIV	HC	PLHIV vs HC	PLHIV- no ABC	PLHIV - ABC	non-ABC vs ABC
n	**208**	**56**	**P-value**	**114**	**94**	**P-value**
d-dimer	219.1 [160.6, 334.8]	170.5 [122.0, 307.2]	0.002#	213.4 [158.6, 335.5]	221.9 [162.6, 334.2]	0.673
Fibrinogen	3.4 [2.8, 4.0]	3.2 [2.8, 4.1]	0.746	3.4 [2.8, 4.0]	3.3 [2.8, 4.0]	0.632
Prothrombin	109.0 [89.3, 135.1]	135.1 [102.4, 162.5]	<0.001#	106.0 [87.1, 135.1]	113.4 [94.8, 134.8]	0.305
Protein S	91.4 [80.7, 110.3]	86.9 [72.7, 112.4]	0.121	91.7 [81.7, 109.4]	91.4 [78.2, 110.4]	0.937
vWf	42.7 [31.8, 59.4]	32.5 [24.4, 43.9]	<0.001#	42.3 [31.6, 61.6]	43.0 [32.4, 57.3]	0.623
iFABP	499.6 [263.0, 717.1]	242.5 [112.9, 376.9]	<0.001#	576.2 [287.4, 897.2]	400.6 [240.5, 619.2]	0.004#
hsCRP	1446 [608, 2735]	651 [205, 1179]	<0.001#	1558 [624, 3285]	1192 [594, 2249]	0.337
sCD14	2139 [1778, 2625]	1789 [1502, 2071]	<0.001#	2063 [1745, 2591]	2236 [1830, 2684]	0.193
sCD163	716.2 [525.6, 898.8]	517.3 [410.7, 578.1]	<0.001#	765.8 [581.6, 916.1]	634.5 [487.8, 858.3]	0.019
Thrombin generation						
ETP	86.6 [78.6, 99.6]	93.0 [83.3, 105.0]	0.011#	83.7 [74.7, 97.6]	90.2 [82.7, 101.6]	0.001#
Lag time	2.0 [1.7, 2.3]	2.0 [1.7, 2.0]	0.382	2.0 [1.7, 2.3]	2.0 [1.7, 2.3]	0.557
TTP	4.4 [4.0, 5.0]	4.3 [3.5, 5.0]	0.035	4.3 [4.0, 5.0]	4.4 [4.0, 5.0]	0.566
Peak	82.6 [70.0, 95.8]	88.1 [72.3, 101.5]	0.031	79.9 [65.2, 96.0]	85.8 [76.1, 95.7]	0.083
VI	78.1 [56.2, 102.7]	97.3 [64.0, 123.5]	0.001	76.4 [53.6, 102.5]	78.3 [61.2, 102.5]	0.566
Protein C activity						
LagT-TM	1.7 [1.4, 2.0]	1.7 [1.4, 2.0]	0.704	1.7 [1.4, 2.3]	1.7 [1.7, 2.0]	0.645
TTP-TM	3.7 [3.3, 4.0]	3.7 [3.3, 4.0]	0.641	3.7 [3.3, 4.0]	3.7 [3.3, 4.0]	0.982
ETP-TM	40.6 [27.9, 52.0]	50.1 [32.8, 76.2]	<0.001	41.0 [28.0, 51.1]	39.7 [26.3, 52.6]	0.706
PEAK-TM	49.6 [31.8, 62.4]	57.1 [39.8, 82.5]	0.002	50.9 [33.2, 61.6]	47.9 [29.9, 62.5]	0.553
VI-TM	59.8 [38.6, 82.5]	71.9 [51.8, 112.5]	<0.001	62.1 [39.8, 85.4]	59.6 [33.2, 77.5]	0.509
nETP-TM_{SR}	0.9 [0.7, 1.2]	1.2 [0.8, 1.5]	0.001	0.9 [0.7, 1.2]	0.9 [0.6, 1.2]	0.183
nPEAK-TM_{SR}	0.9 [0.8, 1.1]	1.1 [0.9, 1.3]	<0.001	1.0 [0.8, 1.1]	0.9 [0.8, 1.1]	0.185
nVI-TM_{SR}	1.0 [0.9, 1.2]	1.1 [0.9, 1.2]	0.43	1.1 [0.9, 1.2]	1.0 [0.9, 1.2]	0.366

High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation (TG) was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR). P-values were calculated using Mann-Whitney U test. Circulating factors and ETP as the primary outcome for TG were corrected for multiple testing using Benjamini-Hochberg procedure (FDR).

Ex vivo coagulation capacity by thrombin generation

Next, *ex vivo* coagulation capacity was determined by *ex vivo* calibrated thrombin generation in plasma. Overall, calibrated thrombin generation showed lower thrombin generation, most notably reduced endogenous thrombin potential (ETP), our primary outcome regarding TG, in PLHIV compared to uninfected controls (table 2, FDR-corrected). In addition PLHIV experienced reduced peak thrombin formation and increased lag time PLHIV compared to HIV-negative controls (data shown in table 2). This difference in ETP persisted (FDR-corrected) only in a subgroup analysis of participants aged 40 years and above (Supplemental table 2), but disappeared in the subgroup of only men (supplemental table 3). However, after correction for age and sex, none of the thrombin generation parameters were significantly different between both cohorts (supplemental figure 1). Most women used oral contraceptive during sampling, which may account for the strong changes in TG parameters when stratifying for sex in the uninfected cohort [29]. Within the PLHIV cohort, no clear association was found between ETP and CD4 nadir, HIV duration or current CD4 count (Figure 1; supplemental table 5). Higher current CD4 count, and CD4-recovery were correlated with increased peak thrombin and ETP (data shown in supplemental table 5), whereas HIV-RNA zenith and HIV duration were associated with decreased protein C activity (nETP-TMsr; supplemental table 4). With the exception of nETP-TMsr, we found no associations between smoking and TG parameters (supplemental table 5).

Inflammation and ex vivo coagulation capacity

While inflammatory markers and D-dimer correlated positively, ETP did not correlate with any of the included inflammatory markers. Only lag time and time-to-peak correlated with sCD14, sCD163 and hsCRP (data shown in figure 1). Moreover, sCD163 showed a negative correlation with Protein C activity (nETP-TMsr; R -0.2, P<0.05, figure 1).

Increased TG in abacavir- vs non-abacavir-treated PLHIV

Next, we explored whether different antiretroviral drugs were associated with TG. There was no association between the use of a protease inhibitor (PI), integrase inhibitor (INSTI) or non-nucleoside reverse transcriptase inhibitor (NNRTI; figure 2) with ETP or any of the other TG parameters. In contrast, abacavir-use was associated with an increased ETP (non-abacavir: 83.7% [74.7, 97.6] vs 90.2% [82.7, 101.6], p= 0.001; figure 2 and table 2). Also, there was a trend towards higher peak thrombin concentrations in the abacavir-group (79.9% [65.2, 96.0] vs 85.8% [76.1, 95.7] p= 0.083; table 2). This effect of abacavir on ETP was independent of age, sex and inflammation (ETP: B= 8.00, p=0.006; Supplemental table 5). Tenofovir difumarate (TDF) use was the most prescribed alternative for abacavir. As a consequence, TDF was associated with a reduced ETP in our cohort (B=-7.86, p=-0.006). However, in multivariate analysis, the effect of abacavir appeared to be larger than TDF on ETP. Therefore, we attributed the effect

of NRTIs on ETP to abacavir in this study. To a similar extent, we could not clearly discriminate between lamivudine and abacavir in this cohort, as this was the most prescribed combination of NRTIs (all data from multivariate analyses are shown in supplemental table 4).

As INSTI-use was high in the abacavir-group and showed a trend towards lower ETP, we corrected for INSTI use (B=-5.79, p=0.061) and the effect of abacavir on ETP became more pronounced (ETP: B=8.85, P=0.002).

The abacavir-associated increase in ETP was not due to decreased protein C activity, a known inhibitor of plasmatic coagulation, as nETPsr was comparable between groups even after correction for age, sex and inflammation (abacavir: B=-0.895, p=0.37).

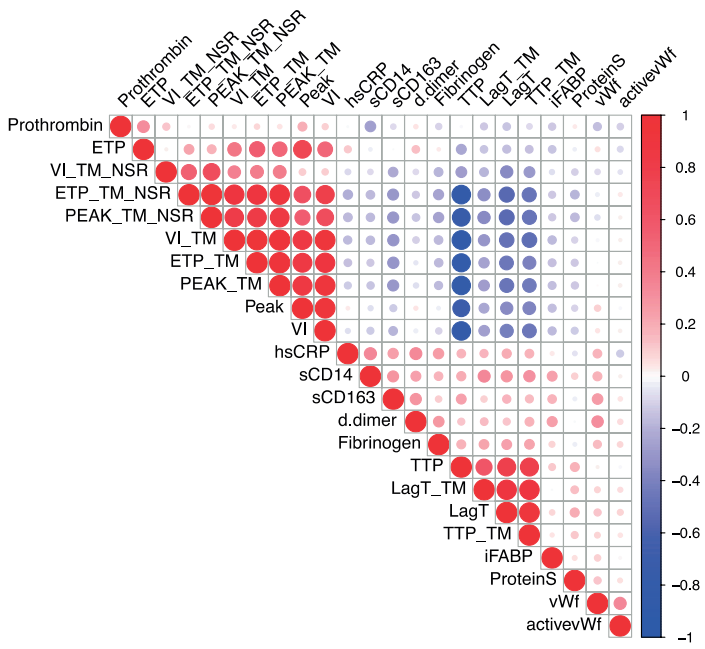


Figure 1. Spearman correlation matrix. Spearman's Correlation coefficients are shown in color and size of circles. Parameters are clustered using hierarchal clustering based on coefficients. Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

In most analyses on abacavir-associated cardiovascular risk in treated PLHIV, there was a discrepancy between current use, past use (>6 months) and cumulative abacavir-use (reviewed by Llibre et al [30]). Current-use of abacavir was more strongly associated to ETP in our cohort than cumulative use. When analysis was restricted to current abacavir users, cumulative exposure (in days) was not correlated with ETP (B=0.597, p=0.552).

When restricting the analysis to non-abacavir users, PLHIV had a decreased ETP, with increased lag time and a more pronounced protein C activity (table 2 and supplemental table 6) compared to uninfected controls. In summary, our data show that abacavir-use is associated with an increase in ex vivo coagulation capacity reflected by increased TG, whereas overall, PLHIV on a non-abacavir regimen showed a decreased coagulation capacity measured by TG.

Mechanisms of decreased TG

Decreased TG and increased D-dimer concentrations suggest consumption of coagulation factors. Indeed, prothrombin concentrations were decreased in PLHIV (figure 3c), even after correction for age and sex (PLHIV: 109.0% [89.3, 135.1], HC: 135.1% [102.4,162.5], p<0.001; supplemental table 1). While prothrombin concentrations were decreased in PLHIV compared to controls, fibrinogen concentrations (PLHIV: 3.4mg/mL [2.8,4.0], HC: 3.2mg/mL [2.8, 4.1], p=0.746) did not differ between groups (figure 3a,c). Furthermore, prothrombin was strongly correlated with ETP parameters in both PLHIV and uninfected controls (figure 3d and supplemental table 6) suggesting that the reduced concentration of prothrombin may underlie the observed decrease in TG in PLHIV. Reduced production of prothrombin and other coagulation factors (by the liver) was deemed unlikely due to similar concentrations of protein S (PLHIV: 91.7% [81.7, 109.4], HC: 86.9% [72.7,112.4], p=0.163, figure 3e) and fibrinogen. No difference in prothrombin or fibrinogen could be observed when PLHIV were stratified based on abacavir-use (Table 2).

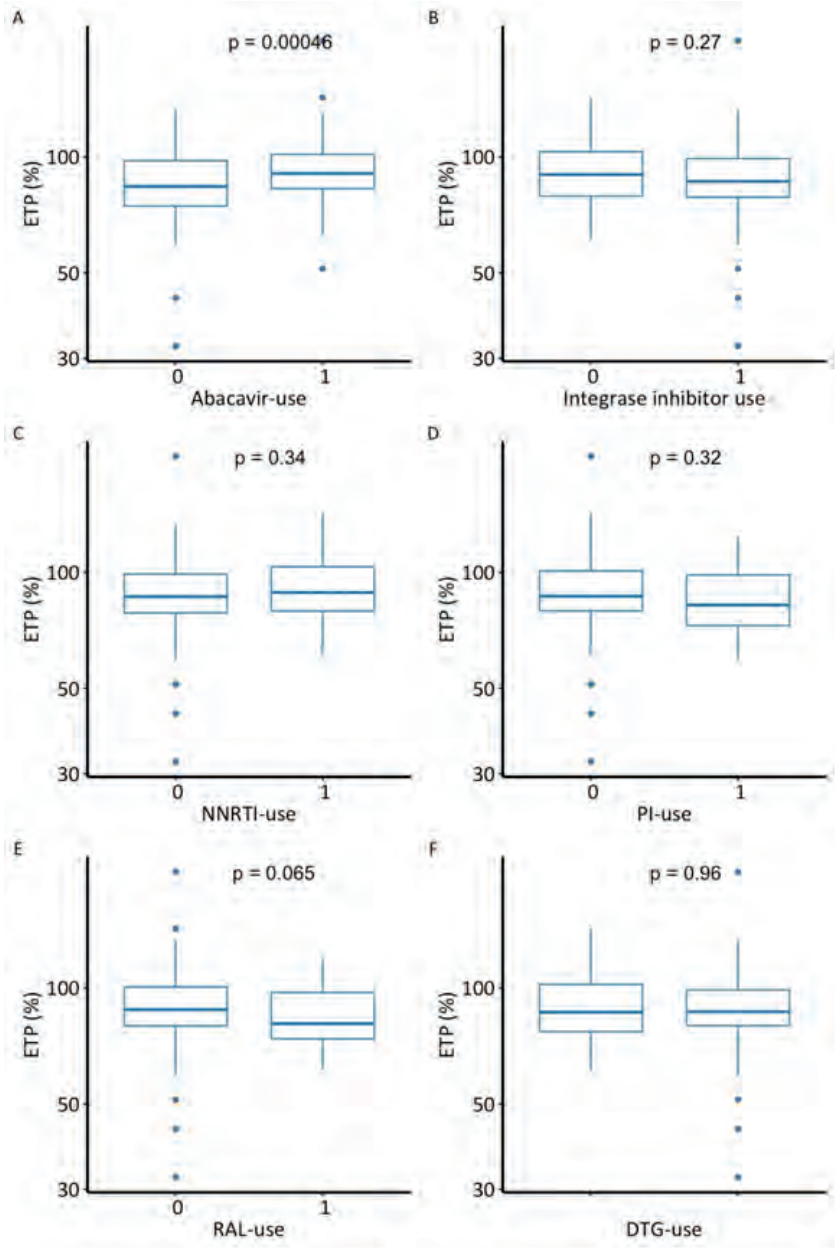


Figure 2. Endogenous thrombin potential stratified per cART-use. Endogenous thrombin potential (ETP; nM×min) were normalized as the percentage (%) of that of normal pooled plasma (NPP) tested in the same run. ABC: abacavir, INSTI: Integrase inhibitor, PI: Protease inhibitor, NNRTI: non-nucleoside reverse transcriptase inhibitor, RAL: raltegravir, DTG: Dolutegravir Values were compared using Mann-Whitney U test. Data are depicted according to Tukey. Comparisons are corrected for multiple testing using Benjamini-Hochberg procedure. ETP remained significantly higher in ABC users compared to non-ABC after FDR correction.

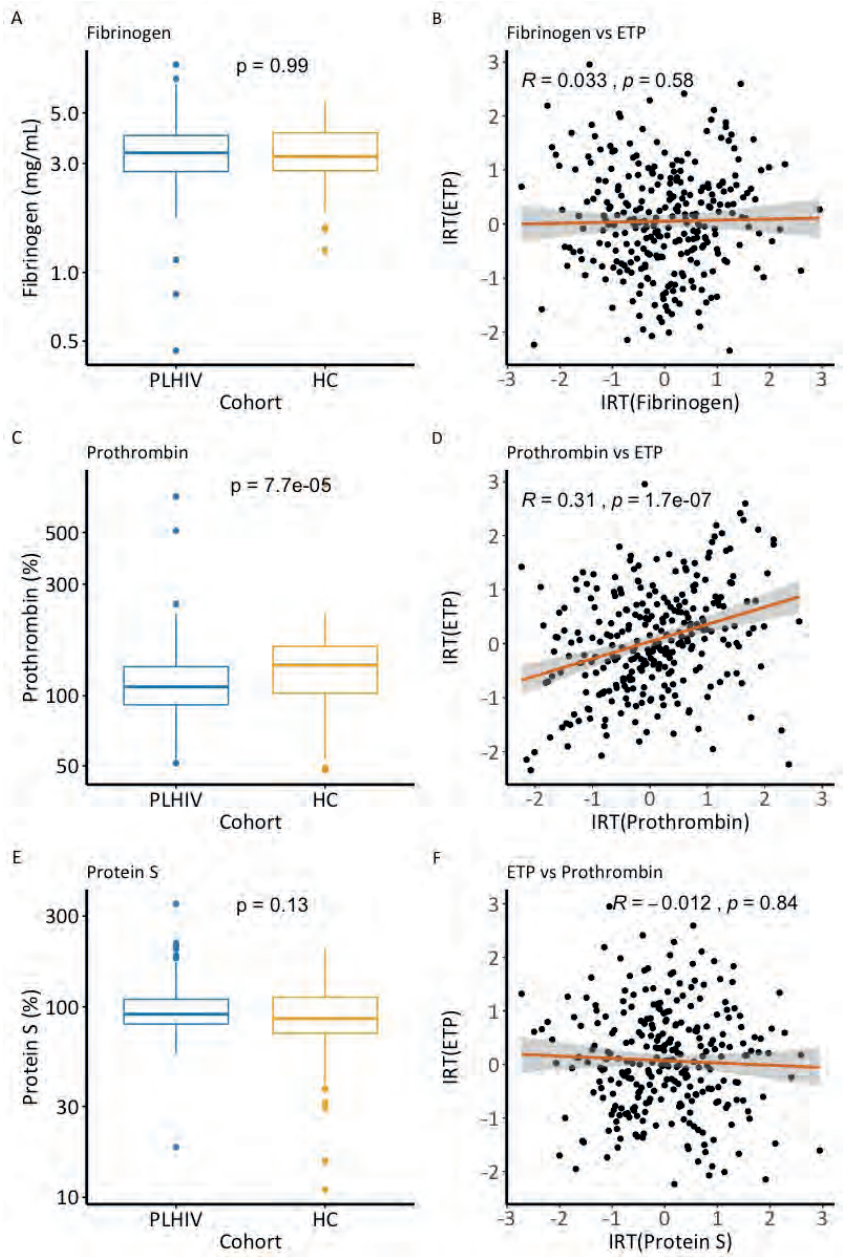


Figure 3. Coagulation factors in PLHIV and healthy controls. (**A**) Fibrinogen concentration (mg/mL) in PLHIV and HC. (**B**) Correlation between inverse rank-based transformed (IRT) Endogenous thrombin potential (ETP; % of normal pooled plasma (NPP)) and fibrinogen (**C**) Prothrombin concentrations (% of NPP) in PLHIV and controls. (**D**) Correlation plot for IRT prothrombin and IRT ETP. (**E**) Protein S concentrations (% of NPP) in PLHIV and controls. (**F**) IRT Protein S and IRT ETP correlation plot. PLHIV: people living with HIV, HC: healthy controls. ETP: Endogenous thrombin production. Correlations coefficients are Pearson's coefficients after inverse-rank based correction (IRT) for normalization. Comparisons between cohorts were done using Mann-Whitney U-test.

Discussion

In a cohort of virally suppressed PLHIV on chronic cART, we show that abacavir-use was associated with increased TG compared with non-abacavir regimens. Overall, PLHIV exhibited reduced TG compared to HIV-uninfected controls, which was associated with lower plasma concentrations of prothrombin. PLHIV had higher concentrations of inflammatory markers, which correlated with D-dimer concentrations, but not with TG parameters.

TG is a diagnostic tool for hypercoagulability states. This is not limited to venous thrombosis, and may also include arterial thrombosis[31]. The use of abacavir has been linked to an increased risk for acute cardiovascular events in different studies [10-13], but not in all [14,15]. In contrast, abacavir use does not appear to be associated with an increased incidence of venous thrombosis[5]. Increased platelet activation was suggested to underlie the excess cardiovascular risk in users of abacavir [32-34], even though increased platelet activation was not observed in all studies [35]. A recent study found that abacavir led to an inflammatory and prothrombotic endothelial phenotype promoting *in vivo* platelet activation via endothelial microparticles (EMP) [36]. The increased TG in abacavir-use could also be caused by endothelial dysfunction and possible prothrombotic EMPs[36,37]. In addition, thrombin directly activates platelets, so our finding of increased TG capacity in abacavir users may as such contribute to increased platelet activation and CVD [38]. In contrast to our results, a study by Jong et al [24] showed no difference in TG for abacavir-use, but only with a limited sample size of 27 individuals in the abacavir-group.

While inflammation was increased in PLHIV, we found no increase in TG. Moreover, the subgroup analysis of non-abacavir users showed a slight decrease in TG in PLHIV compared to uninfected controls. This observation is in line with earlier observations showing decreased TG in long-term treated PLHIV [25]. In this study, we confirm these results using a cohort of long-term treated PLHIV on more recently recommended cART regimens. Regarding possible mechanisms, consumption of clotting factors could play a role, as prothrombin concentrations were decreased with a concurrent increase in D-dimer in the total PLHIV group. Fibrinogen concentrations were similar across all groups, but fibrinogen is a well-known acute phase protein and could therefore reflect inflammation rather than coagulation capacity [39]. Furthermore, decreased production of (pro)coagulation factors was deemed less likely as other factors produced by the liver such as fibrinogen and protein S were similar across groups.

More recent papers have used *in silico* TG as a proxy for plasmatic coagulation potential and showed that this calculated TG correlate with development of CVD[40]. Furthermore, in the SMART study, in silico TG was lower in PLHIV without ART and ongoing viral replication compared to cART treated PLHIV [41,42]. While D-dimer mirrors coagulation and fibrinolysis *in vivo*, *in vitro* TG reflects actual hemostatic potential of the plasmatic coagulation pathways [22]. We confirmed that D-dimers indeed correlated with endothelial activation (e.g. vWF) and inflammation (eg. sCD163), but found no such relation with TG parameters in PLHIV. This suggests that increased D-dimer concentrations are primarily influenced by increased provoking signals for coagulation, such as endothelial activation and inflammation during HIV infection. However, increased plasmatic coagulation capacity could still play a role in cardiovascular disease in PLHIV, as was shown by a case-control study using in silico TG [42]. A significant role for plasmatic coagulation and increased TG has been shown in clinical studies including uninfected patients at risk for cardiovascular disease [31]. As PLHIV are known for increased inflammation and endothelial activation, an abacavir-associated potentiation of the plasmatic coagulation capacity could therefore still result in increased risk for arterial thrombosis.

Interestingly, protein C activity was higher in PLHIV and correlated with inflammation (sCD163) thereby reducing the overall plasmatic coagulation capacity. This rebalancing of the anti- and procoagulant pathway during HIV infection mimics the rebalancing of hemostasis seen in liver disease [43]. Monocyte and macrophage activation (sCD14 and sCD163) correlated with lag time and TTP. These parameters are mostly influenced by an increase in Tissue factor pathway inhibitor (TFPI) [44]. This TFPI is known to be increased in PLHIV [45]. Even though endothelial cells and platelets mainly produce TFPI, monocytes are known producers of TFPI too and could increase production upon activation [44].

Limitations and strengths

The cross-sectional design of our study does not allow to draw causal inferences. Furthermore, the independent increase in TG seen in abacavir treatment could be affected through indication bias whereby treatment selection is influenced by patient characteristics. Yet, in our case abacavir was preferably given to people with a decreased cardiovascular risk profile, which was reflected in our cohort with a trend towards lower CVD risk factors in the abacavir-treated group. Second, as TDF was the most prescribed alternative to abacavir, we cannot fully rule out that TDF has an inhibitory effect on TG. Yet, it will not change the conclusions that abacavir is associated with increased TG compared to non-abacavir users. To similar extent we cannot exclude the possibility that lamivudine is involved in this difference as lamivudine was prescribed concurrently to abacavir in the majority of patients. Third, we could not correlate TG parameters with clinical outcomes as was performed in the study reporting on in silico TG. While in silico TG can be calculated from plasma markers measured in (long-term) stored EDTA plasma, our functional assay of calibrated TG (CAT) requires citrated plasma, which is not regularly collected in large-scale cohorts. Actual measurement of thrombin formation using the CAT method is more precise than the modeling of the *in silico* TG method, because CAT also includes possible unknown confounders such as cART-use or other unknown factors. Therefore, CAT better reflects actual hemostatic potential in complex

disease states such as an HIV infection. Furthermore, due to lower number of women in our study, generalizability of our findings to women is limited and warrants further study. Our cohort only consists of virally suppressed PLHIV on long-term treatment and reflects the current HIV-infected population. Thus, our data cannot be used to draw conclusions about patients that are still severely immune compromised or with ongoing viral replication.

In conclusion, abacavir-use was associated with increased TG and this increase could serve as an additional factor in the reported increase in thrombotic events during abacavir-use. Increased exposure to triggers that propagate coagulation, such as inflammation, and endothelial activation likely underlie increased D-dimer concentrations found in most PLHIV.

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Supplementary Table 1. Linear regression using age and sex as co-factors in multivariate analysis.

	Coefficient (univariate)	Coefficient(corrected for age-sex)
d-dimer	52.75 (3.73 to 101.77, p=0.035)	6.87 (-61.05 to 74.78, p=0.842)
iFABP	289.4 (123.5 to 455.3, p=0.001)	278.5 (95.3 to 461.6, p=0.003)
hsCRP	1595 (210 to 2981, p=0.024)	1863 (324 to 3402, p=0.018)
Fibrinogen	0.07 (-0.17 to 0.32, p=0.557)	-0.07 (-0.43 to 0.29, p=0.707)
vWf	13207 (5679 to 20735, p=0.001)	7120 (-3965 to 18204 p=0.207)
sCD14	423.8 (213.3 to 634.5, p<0.001)	319.9 (886.8 to 551.2, p=0.007)
sCD163	215.4 (120.1 to 310.6, p<0.001)	152.74(49.3 to 256.2, p=0.004)
Prothrombin	-21.39 (-37.29 to -5.49, p=0.009)	-23.40 (-43.15 to -3.64, p=0.020)
ProteinS	6.55 (-1.70 to 14.80, p=0.119)	-3.21 (-15.00 to 8.57, p=0.592)
ETP	-6.37 (-11.06 to -1.68, p=0.008)	-4.86 (-11.39 to 1.67, p=0.144)
LagT	0.06 (-0.06 to 0.18, p=0.336)	-0.06 (-0.23 to 0.12, p=0.525)
TTP	0.25 (0.04 to 0.47, p=0.022)	0.06 (-0.24 to 0.36, p=0.708)
Peak	-6.50 (-11.84 to -1.16, p=0.017)	-2.25 (-9.60 to 5.10, p=0.547)
VI	-19.30 (-28.72 to -9.87, p<0.001)	-7.50 (-19.93 to 4.94, p=0.236)
LagT_TM	0.02 (-0.11 to 0.14, p=0.799)	-0.07 (-0.25 to 0.11, p=0.454)
TTP_TM	0.08 (-0.06 to 0.22, p=0.270)	-0.06 (-0.26 to 0.14, p=0.569)
ETP_TM	-12.27 (-18.39 to -6.15, p<0.001)	-5.36 (-13.50 to 2.79, p=0.196)
ETP_TM_NSR	-0.20 (-0.30 to -0.09, p<0.001)	-0.08 (-0.23 to 0.06, p=0.259)
PEAK_TM	-12.18 (-18.97 to -5.38, p<0.001)	-3.28 (-12.34 to 5.77, p=0.476)
VI_TM	-20.94 (-31.16 to -10.72, p<0.001)	-5.43 (-18.65 to 7.79, p=0.419)
PEAK_TM_NSR	-0.15 (-0.23 to -0.08, p<0.001)	-0.08 (-0.18 to 0.03, p=0.155)
VI_TM_NSR	-0.01 (-0.07 to 0.06, p=0.883)	0.01 (-0.09 to 0.11, p=0.847)

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von Willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR).

Supplementary Table 2. Subgroup analysis of individuals aged 40 years and above

	PLHIV (n=173)	HC (n=22)	p
age (mean (SD))	54.8 (7.7)	59.4 (10.3)	0.013
BMI (median [IQR])	24.4 [22.4, 26.6]	24.5 [22.3, 26.9]	0.954
d.dimer (median [IQR])	233.7 [166.2, 339.6]	333.0 [187.0, 391.9]	0.167
Plasma vWf (median [IQR])	4550 [3442, 6248]	3453 [2341, 3923]	0.003#
iFABP (median [IQR])	521.0 [286.2, 748.9]	371.8 [317.0, 545.6]	0.132
hsCRP (median [IQR])	1504 [608, 3099]	812.6 [382.2, 1671.0]	0.073
sCD14 (median [IQR])	2207 [1787, 2659]	1952 [1741, 2134]	0.066
sCD163 (median [IQR])	740.9 [540.6, 904.7]	516.8 [442.7, 684.2]	0.011#
Fibrinogen (median [IQR])	3.4 [2.8, 4.0]	4.0 [3.0, 4.5]	0.074
Prothrombin (median [IQR])	109.0 [88.1, 136.3]	161.5 [134.9, 180.2]	<0.001#
Protein S (median [IQR])	91.7 [81.7, 111.9]	83.7 [71.3, 118.0]	0.325
ETP (median [IQR])	86.6 [78.7, 101.0]	98.6 [94.3, 105.5]	0.014#
LagT (median [IQR])	2.0 [1.7, 2.3]	2.0 [1.7, 2.4]	0.707
TTP (median [IQR])	4.3 [4.0, 5.0]	4.7 [3.7, 5.0]	0.938
Peak (median [IQR])	82.5 [68.8, 95.8]	90.1 [80.1, 103.4]	0.1
VI (median [IQR])	78.1 [56.0, 102.0]	90.2 [71.2, 108.8]	0.134
LagT_TM (median [IQR])	1.7 [1.4, 2.0]	2.0 [1.7, 2.0]	0.165
TTP_TM (median [IQR])	3.7 [3.3, 4.0]	4.0 [3.6, 4.0]	0.296
ETP_TM (median [IQR])	41.1 [27.0, 51.7]	47.9 [41.0, 63.4]	0.066
PEAK_TM (median [IQR])	50.1 [31.9, 61.8]	52.4 [46.2, 68.2]	0.241
VI_TM (median [IQR])	59.6 [37.4, 82.3]	65.2 [55.6, 83.8]	0.207
ETP_TM_NSR (median [IQR])	0.9 [0.7, 1.2]	0.9 [0.7, 1.3]	0.558
PEAK_TM_NSR (median [IQR])	0.9 [0.8, 1.1]	0.9 [0.8, 1.2]	0.494
VI_TM_NSR (median [IQR])	1.0 [0.9, 1.3]	1.0 [0.9, 1.3]	0.97

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR). Circulating factors and ETP as the primary outcome for TG were corrected for multiple testing using Benjamini-Hochberg procedure (FDR). All remaining TG parameters were treated as exploratory parameters # Significantly different between groups after FDR-correction.

Supplementary Table 3. Subgroup analysis restricting to males only

	PLHIV (n=190)	HC (n=34	p
AGE (mean (SD))	51.2 (11.0)	39.7 (17.7)	<0.001
BMI (median [IQR])	24.1 [22.0, 25.9]	23.9 [21.9, 25.4]	0.89
d.dimer (median [IQR])	216.5 [158.0, 334.2]	166.9 [115.2, 275.4]	0.019#
Plasma vWf (median [IQR])	42675 [31896, 58881]	31559 [26013, 45904]	0.011#
iFABP (median [IQR])	478.1 [245.5, 694.7]	268.3 [134.6, 397.5]	0.003#
hsCRP (median [IQR])	1451 [607, 2688]	675 [166, 1309]	0.003#
sCD14 (median [IQR])	2139 [1779, 2630]	1851 [1614, 2172]	0.007#
sCD163 (median [IQR])	700.2 [516.8, 900.0]	532.1 [418.3, 620.5]	0.002#
Fibrinogen (median [IQR])	3.4 [2.8, 4.0]	3.3 [2.8, 4.0]	0.615
Prothrombin (median [IQR])	109.1 [89.3, 136.7]	136.7 [110.6, 162.1]	0.003#
ProteinS (median [IQR])	91.7 [80.3, 111.3]	99.7 [79.9, 133.6]	0.315
ETP (median [IQR])	86.6 [78.6, 99.2]	86.8 [80.5, 98.7]	0.961
LagT (median [IQR])	2.0 [1.7, 2.3]	2.0 [2.0, 2.3]	0.052
TTP (median [IQR])	4.4 [4.0, 5.0]	4.7 [4.0, 5.0]	0.329
Peak (median [IQR])	82.4 [68.7, 95.4]	78.4 [66.3, 96.8]	0.588
VI (median [IQR])	76.8 [55.9, 100.7]	76.1 [56.2, 104.6]	0.813
LagT_TM (median [IQR])	1.7 [1.4, 2.0]	2.0 [1.7, 2.0]	0.029
TTP_TM (median [IQR])	3.7 [3.3, 4.0]	4.0 [3.7, 4.0]	0.082
ETP_TM (median [IQR])	40.6 [27.7, 52.0]	43.5 [30.0, 50.5]	0.764
PEAK_TM (median [IQR])	49.5 [31.8, 62.2]	49.9 [37.9, 62.2]	0.898
VI_TM (median [IQR])	59.6 [38.2, 80.2]	60.2 [44.2, 81.7]	0.691
ETP_TM_NSR (median [IQR])	0.9 [0.7, 1.2]	0.9 [0.7, 1.3]	0.594
PEAK_TM_NSR (median [IQR])	0.9 [0.8, 1.1]	1.0 [0.8, 1.2]	0.347
VI_TM_NSR (median [IQR])	1.1 [0.9, 1.2]	1.1 [0.9, 1.2]	0.711

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR). Circulating factors and ETP as the primary outcome for TG were corrected for multiple testing using Benjamini-Hochberg procedure (FDR). All remaining TG parameters were treated as exploratory parameters # Significantly different between groups after FDR-correction.

Supplementary Table 4. Univariate and multivariate analysis of circulating markers

	Univariate	Model 1 (Age-sex)	model (age-sex-sCD14)
ETP			
Prothrombin	4.45 (0.79 to 8.11, p=0.018)	5.81 (1.92 to 9.70, p=0.004)	5.90 (2.02 to 9.79, p=0.003)
Fibrinogen	-6.28 (-9.73 to -2.82, p<0.001)	-6.56 (-10.26 to -2.87, p=0.001)	-6.41 (-10.13 to -2.69, p=0.001)
sCD163	1.96 (-4.54 to 8.46, p=0.553)	-2.56 (-10.29 to 5.17, p=0.513)	-1.54 (-9.41 to 6.33, p=0.700)
sCD14	-1.90 (-11.49 to 7.69, p=0.697)	-8.07 (-19.47 to 3.33, p=0.164)	-8.07 (-19.47 to 3.33, p=0.164)
hsCRP	1.48 (-0.61 to 3.58, p=0.165)	0.43 (-1.99 to 2.84, p=0.727)	0.77 (-1.72 to 3.25, p=0.543)
iFABP	-2.35 (-5.13 to 0.43, p=0.097)	-2.87 (-5.98 to 0.24, p=0.071)	-2.84 (-5.94 to 0.26, p=0.072)
PEAK			
Prothrombin	1.03 (-3.32 to 5.39, p=0.640)	2.16 (-2.58 to 6.90, p=0.370)	2.28 (-2.47 to 7.02, p=0.345)
Fibrinogen	-5.23 (-9.39 to -1.08, p=0.014)	-6.15 (-10.65 to -1.65, p=0.008)	-5.96 (-10.50 to -1.43, p=0.010)
sCD163	-4.71 (-12.30 to 2.87, p=0.222)	-8.11 (-17.20 to 0.98, p=0.080)	-7.18 (-16.45 to 2.09, p=0.128)
sCD14	-6.34 (-17.53 to 4.86, p=0.266)	-9.07 (-22.58 to 4.45, p=0.187)	-9.07 (-22.58 to 4.45, p=0.187)
hsCRP	0.48 (-2.00 to 2.97, p=0.701)	-0.24 (-3.13 to 2.65, p=0.870)	0.18 (-2.79 to 3.15, p=0.906)
iFABP	-2.99 (-6.23 to 0.26, p=0.071)	-2.99 (-6.69 to 0.70, p=0.112)	-2.97 (-6.65 to 0.72, p=0.114)
TTP			
Prothrombin	0.10 (-0.08 to 0.29, p=0.283)	0.08 (-0.12 to 0.29, p=0.409)	0.08 (-0.12 to 0.27, p=0.457)
Fibrinogen	0.15 (-0.02 to 0.33, p=0.089)	0.23 (0.04 to 0.41, p=0.018)	0.21 (0.02 to 0.40, p=0.027)
sCD163	0.51 (0.20 to 0.82, p=0.001)	0.49 (0.12 to 0.86, p=0.010)	0.42 (0.04 to 0.79, p=0.029)
sCD14	0.69 (0.23 to 1.15, p=0.003)	0.63 (0.08 to 1.19, p=0.024)	0.63 (0.08 to 1.19, p=0.024)
hsCRP	0.12 (0.02 to 0.22, p=0.023)	0.09 (-0.02 to 0.21, p=0.114)	0.07 (-0.05 to 0.19, p=0.264)
iFABP	0.07 (-0.07 to 0.20, p=0.336)	0.06 (-0.09 to 0.22, p=0.420)	0.06 (-0.09 to 0.21, p=0.429)
LagTime			
Prothrombin	-0.00 (-0.11 to 0.10, p=0.933)	-0.03 (-0.15 to 0.09, p=0.611)	-0.04 (-0.15 to 0.07, p=0.501)
Fibrinogen	0.15 (0.05 to 0.25, p=0.004)	0.20 (0.09 to 0.30, p<0.001)	0.18 (0.08 to 0.28, p=0.001)
sCD163	0.25 (0.07 to 0.43, p=0.007)	0.17 (-0.04 to 0.39, p=0.119)	0.09 (-0.12 to 0.30, p=0.395)
sCD14	0.64 (0.38 to 0.90, p<0.001)	0.62 (0.32 to 0.93, p<0.001)	0.62 (0.32 to 0.93, p<0.001)
hsCRP	0.10 (0.05 to 0.16, p=0.001)	0.08 (0.02 to 0.15, p=0.015)	0.06 (-0.01 to 0.12, p=0.087)
iFABP	0.01 (-0.07 to 0.09, p=0.809)	0.02 (-0.07 to 0.11, p=0.704)	0.02 (-0.07 to 0.10, p=0.724)
nETPTMsr			
Prothrombin	-0.07 (-0.16 to 0.01, p=0.105)	-0.07 (-0.16 to 0.01, p=0.096)	-0.07 (-0.16 to 0.01, p=0.102)
Fibrinogen	-0.06 (-0.16 to 0.03, p=0.188)	-0.08 (-0.18 to 0.02, p=0.113)	-0.08 (-0.18 to 0.02, p=0.111)
sCD163	-0.21 (-0.37 to -0.04, p=0.014)	-0.20 (-0.38 to -0.02, p=0.032)	-0.20 (-0.39 to -0.02, p=0.032)
sCD14	-0.19 (-0.43 to 0.05, p=0.118)	-0.02 (-0.30 to 0.26, p=0.885)	-0.02 (-0.30 to 0.26, p=0.885)
hsCRP	-0.07 (-0.12 to -0.02, p=0.009)	-0.06 (-0.11 to -0.00, p=0.033)	-0.06 (-0.11 to -0.00, p=0.038)
iFABP	-0.01 (-0.08 to 0.06, p=0.817)	-0.01 (-0.08 to 0.06, p=0.802)	-0.01 (-0.08 to 0.06, p=0.812)

IRT_ETP				
Prothrombin	0.22 (0.03 to 0.41, p=0.026)	0.30 (0.10 to 0.49, p=0.003)	0.27 (0.08 to 0.46, p=0.006)	
Fibrinogen	-0.30 (-0.48 to -0.12, p=0.001)	-0.31 (-0.49 to -0.12, p=0.001)	-0.28 (-0.46 to -0.10, p=0.003)	
sCD163	0.19 (-0.15 to 0.52, p=0.268)	-0.06 (-0.45 to 0.32, p=0.754)	0.13 (-0.26 to 0.52, p=0.509)	
sCD14	-0.07 (-0.57 to 0.42, p=0.779)	-0.47 (-1.04 to 0.10, p=0.104)	0.42 (0.14 to 0.70, p=0.004)	
hsCRP	0.11 (0.00 to 0.22, p=0.049)	0.04 (-0.08 to 0.16, p=0.466)	0.08 (-0.04 to 0.20, p=0.201)	
iFABP	-0.12 (-0.26 to 0.03, p=0.109)	-0.15 (-0.30 to 0.01, p=0.066)	-0.12 (-0.27 to 0.04, p=0.133)	

Linear regression was used for analysis. Data shown as Beta with confidence interval and p-value. Model 1 includes age, time of inclusion and sex as covariates. Model 2 also includes sCD14 as a covariate. High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP; IRT: inverse rank based normalization.

Supplemental Table 5. Univariate and multivariate analysis of thrombin generation parameters (by calibrated thrombin generation).

ETP				
	Univariate	model 1 (age-sex)	model 2 (age-sex-sCD14)	model 3 (age-sex-sCD14-ABC)
Smoking (pckyrs)	-0.07 (-0.19 to 0.05, p=0.255)	-0.11 (-0.25 to 0.04, p=0.148)	-0.10 (-0.25 to 0.05, p=0.178)	-0.11 (-0.25 to 0.04, p=0.148)
HIV duration	0.46 (-0.24 to 1.16, p=0.198)	0.52 (-0.28 to 1.32, p=0.203)	0.50 (-0.31 to 1.30, p=0.226)	0.52 (-0.28 to 1.32, p=0.203)
cART duration	0.52 (-0.40 to 1.44, p=0.264)	0.43 (-0.60 to 1.47, p=0.409)	0.47 (-0.57 to 1.50, p=0.376)	0.43 (-0.60 to 1.47, p=0.409)
HIV RNA zenith	-0.09 (-0.29 to 0.12, p=0.414)	-0.15 (-0.43 to 0.12, p=0.271)	-0.15 (-0.42 to 0.13, p=0.291)	-0.15 (-0.43 to 0.12, p=0.271)
CD4 nadir	1.44 (-1.74 to 4.61, p=0.374)	2.90 (-0.83 to 6.63, p=0.127)	2.86 (-0.94 to 6.65, p=0.139)	2.90 (-0.83 to 6.63, p=0.127)
CD4 count	1.56 (-0.99 to 4.12, p=0.229)	1.55 (-1.44 to 4.53, p=0.307)	1.36 (-1.65 to 4.37, p=0.373)	1.55 (-1.44 to 4.53, p=0.307)
CD4-CD8 ratio	0.47 (-0.99 to 1.94, p=0.522)	0.09 (-1.53 to 1.70, p=0.914)	0.09 (-1.52 to 1.71, p=0.908)	0.09 (-1.53 to 1.70, p=0.914)
CD4 recovery (relative)	7.85 (1.41 to 14.29, p=0.017)	9.30 (2.05 to 16.55, p=0.012)	8.68 (1.33 to 16.03, p=0.021)	9.30 (2.05 to 16.55, p=0.012)
INSTI	-2.99 (-8.36 to 2.38, p=0.273)	-3.63 (-9.77 to 2.52, p=0.245)	-4.18 (-10.35 to 1.99, p=0.182)	-3.63 (-9.77 to 2.52, p=0.245)
Raltegravir	-4.95 (-11.49 to 1.59, p=0.137)	-4.98 (-12.79 to 2.82, p=0.209)	-5.75 (-13.60 to 2.10, p=0.150)	-4.98 (-12.79 to 2.82, p=0.209)
Elvitegravir	3.72 (-5.87 to 13.30, p=0.445)	4.19 (-6.49 to 14.87, p=0.440)	3.87 (-6.84 to 14.57, p=0.477)	4.19 (-6.49 to 14.87, p=0.440)
Dolutegravir	-0.42 (-5.52 to 4.68, p=0.871)	-1.25 (-7.06 to 4.57, p=0.673)	-1.19 (-7.03 to 4.64, p=0.687)	-1.25 (-7.06 to 4.57, p=0.673)
PI	-3.01 (-9.98 to 3.96, p=0.395)	-2.70 (-11.03 to 5.64, p=0.524)	-2.78 (-11.11 to 5.56, p=0.512)	-2.70 (-11.03 to 5.64, p=0.524)
NNRTI	2.96 (-2.58 to 8.51, p=0.293)	4.57 (-1.72 to 10.86, p=0.153)	5.27 (-1.06 to 11.60, p=0.102)	4.57 (-1.72 to 10.86, p=0.153)
Tenofovir (TDF)	-8.01 (-12.95 to -3.07, p=0.002)	-7.64 (-13.24 to -2.04, p=0.008)	-7.86 (-13.48 to -2.25, p=0.006)	-7.64 (-13.24 to -2.04, p=0.008)
Lamivudine	8.69 (3.81 to 13.58, p=0.001)	8.53 (3.04 to 14.01, p=0.003)	8.51 (3.02 to 14.01, p=0.003)	8.53 (3.04 to 14.01, p=0.003)
Emtricitabine	-8.41 (-13.35 to -3.47, p=0.001)	-8.67 (-14.24 to -3.11, p=0.002)	-8.98 (-14.55 to -3.41, p=0.002)	-8.67 (-14.24 to -3.11, p=0.002)
Abacavir	8.40 (3.48 to 13.32, p=0.001)	7.68 (2.07 to 13.29, p=0.008)	8.00 (2.38 to 13.63, p=0.006)	NA
PEAK				
Smoking (pckyrs)	-0.12 (-0.26 to 0.02, p=0.090)	-0.14 (-0.31 to 0.03, p=0.115)	-0.13 (-0.30 to 0.04, p=0.143)	-0.12 (-0.30 to 0.05, p=0.160)
HIV duration	0.20 (-0.62 to 1.02, p=0.634)	0.25 (-0.70 to 1.20, p=0.608)	0.21 (-0.75 to 1.17, p=0.667)	0.19 (-0.76 to 1.15, p=0.690)
cART duration	0.51 (-0.57 to 1.58, p=0.352)	0.31 (-0.91 to 1.54, p=0.616)	0.34 (-0.89 to 1.57, p=0.582)	0.43 (-0.80 to 1.67, p=0.490)
HIV RNA zenith	-0.20 (-0.45 to 0.04, p=0.098)	-0.30 (-0.62 to 0.02, p=0.066)	-0.30 (-0.62 to 0.03, p=0.071)	-0.29 (-0.61 to 0.03, p=0.079)

CD4 nadir	1.27 (-2.45 to 4.99, p=0.502)	2.24 (-2.19 to 6.68, p=0.320)	2.03 (-2.49 to 6.55, p=0.377)	2.56 (-2.03 to 7.15, p=0.273)
CD4 count	3.27 (0.30 to 6.23, p=0.031)	3.54 (0.03 to 7.04, p=0.048)	3.43 (-0.11 to 6.96, p=0.057)	3.34 (-0.20 to 6.89, p=0.064)
CD4-CD8 ratio	0.48 (-1.24 to 2.20, p=0.581)	0.22 (-1.70 to 2.14, p=0.821)	0.22 (-1.71 to 2.15, p=0.821)	0.25 (-1.69 to 2.18, p=0.802)
CD4 recovery (relative)	9.78 (2.26 to 17.30, p=0.011)	11.32 (2.75 to 19.90, p=0.010)	10.75 (2.05 to 19.45, p=0.016)	10.69 (1.99 to 19.39, p=0.016)
INSTI	-6.45 (-12.69 to -0.21, p=0.043)	-7.08 (-14.30 to 0.14, p=0.054)	-7.70 (-14.95 to -0.44, p=0.038)	-8.57 (-15.91 to -1.24, p=0.022)
Raltegravir	-5.35 (-13.01 to 2.30, p=0.170)	-6.36 (-15.59 to 2.88, p=0.176)	-7.25 (-16.55 to 2.05, p=0.126)	-6.66 (-16.08 to 2.75, p=0.164)
Elvitegravir	6.83 (-4.36 to 18.02, p=0.230)	8.03 (-4.58 to 20.64, p=0.210)	7.60 (-5.06 to 20.26, p=0.237)	10.58 (-2.64 to 23.81, p=0.116)
Dolutegravir	-3.85 (-9.79 to 2.09, p=0.202)	-4.44 (-11.30 to 2.42, p=0.203)	-4.32 (-11.20 to 2.57, p=0.218)	-7.35 (-14.97 to 0.27, p=0.059)
PI	0.57 (-7.59 to 8.74, p=0.890)	0.06 (-9.82 to 9.95, p=0.990)	-0.06 (-9.96 to 9.84, p=0.990)	0.86 (-9.18 to 10.91, p=0.865)
NNRTI	5.61 (-0.85 to 12.07, p=0.088)	8.17 (0.78 to 15.56, p=0.030)	8.97 (1.53 to 16.40, p=0.018)	10.26 (2.69 to 17.83, p=0.008)
Tenofovir (TDF)	-4.88 (-10.76 to 1.01, p=0.104)	-4.57 (-11.31 to 2.18, p=0.183)	-4.86 (-11.63 to 1.91, p=0.158)	-5.29 (-16.19 to 5.61, p=0.340)
Lamivudine	5.46 (-0.38 to 11.29, p=0.067)	5.12 (-1.51 to 11.76, p=0.129)	5.17 (-1.48 to 11.82, p=0.127)	7.32 (-4.80 to 19.45, p=0.235)
Emtricitabine	-5.95 (-11.83 to -0.07, p=0.047)	-6.25 (-12.96 to 0.45, p=0.067)	-6.64 (-13.37 to 0.09, p=0.053)	-10.60 (-21.94 to 0.73, p=0.067)
Abacavir	4.44 (-1.44 to 10.32, p=0.138)	3.22 (-3.55 to 9.99, p=0.349)	3.60 (-3.20 to 10.41, p=0.297)	NA
TTP				
Smoking (pckyrs)	0.01 (-0.00 to 0.01, p=0.069)	0.01 (-0.00 to 0.01, p=0.067)	0.01 (-0.00 to 0.01, p=0.104)	0.01 (-0.00 to 0.01, p=0.095)
HIV duration	0.02 (-0.02 to 0.05, p=0.324)	0.01 (-0.03 to 0.05, p=0.567)	0.02 (-0.02 to 0.05, p=0.448)	0.01 (-0.02 to 0.05, p=0.460)
cART duration	-0.03 (-0.08 to 0.01, p=0.168)	-0.03 (-0.08 to 0.02, p=0.240)	-0.03 (-0.08 to 0.02, p=0.208)	-0.03 (-0.08 to 0.02, p=0.235)
HIV RNA zenith	0.01 (0.00 to 0.02, p=0.032)	0.02 (0.00 to 0.03, p=0.015)	0.02 (0.00 to 0.03, p=0.015)	0.02 (0.00 to 0.03, p=0.014)
CD4 nadir	0.06 (-0.10 to 0.21, p=0.460)	0.05 (-0.13 to 0.23, p=0.578)	0.08 (-0.10 to 0.27, p=0.376)	0.10 (-0.09 to 0.28, p=0.311)
CD4 count	-0.17 (-0.29 to -0.05, p=0.006)	-0.21 (-0.35 to -0.06, p=0.005)	-0.20 (-0.34 to -0.06, p=0.006)	-0.21 (-0.35 to -0.06, p=0.005)
CD4-CD8 ratio	-0.06 (-0.13 to 0.01, p=0.110)	-0.07 (-0.14 to 0.01, p=0.092)	-0.07 (-0.14 to 0.01, p=0.094)	-0.06 (-0.14 to 0.01, p=0.105)
CD4 recovery (relative)	-0.34 (-0.65 to -0.02, p=0.037)	-0.40 (-0.75 to -0.04, p=0.029)	-0.36 (-0.71 to 0.00, p=0.050)	-0.36 (-0.72 to -0.00, p=0.050)
INSTI	0.23 (-0.03 to 0.49, p=0.079)	0.32 (0.02 to 0.62, p=0.035)	0.36 (0.06 to 0.65, p=0.018)	0.35 (0.05 to 0.65, p=0.022)
Raltegravir	0.16 (-0.16 to 0.48, p=0.318)	0.32 (-0.06 to 0.70, p=0.096)	0.38 (0.01 to 0.76, p=0.046)	0.41 (0.03 to 0.79, p=0.035)
Elvitegravir	-0.52 (-0.98 to -0.06, p=0.028)	-0.51 (-1.03 to 0.00, p=0.051)	-0.48 (-0.99 to 0.03, p=0.067)	-0.47 (-1.01 to 0.07, p=0.085)
Dolutegravir	0.23 (-0.01 to 0.48, p=0.062)	0.24 (-0.05 to 0.52, p=0.099)	0.22 (-0.06 to 0.50, p=0.119)	0.23 (-0.08 to 0.54, p=0.151)
PI	0.16 (-0.18 to 0.50, p=0.349)	0.17 (-0.23 to 0.58, p=0.402)	0.18 (-0.22 to 0.59, p=0.367)	0.21 (-0.20 to 0.62, p=0.307)
NNRTI	-0.25 (-0.52 to 0.02, p=0.070)	-0.33 (-0.63 to -0.02, p=0.034)	-0.38 (-0.68 to -0.08, p=0.015)	-0.37 (-0.69 to -0.06, p=0.018)
Tenofovir (TDF)	-0.08 (-0.33 to 0.16, p=0.516)	-0.08 (-0.36 to 0.19, p=0.551)	-0.06 (-0.34 to 0.22, p=0.659)	0.01 (-0.43 to 0.46, p=0.956)
Lamivudine	0.03 (-0.21 to 0.28, p=0.803)	0.04 (-0.24 to 0.31, p=0.784)	0.03 (-0.24 to 0.30, p=0.821)	-0.13 (-0.63 to 0.37, p=0.610)
Emtricitabine	-0.09 (-0.33 to 0.16, p=0.484)	-0.10 (-0.38 to 0.18, p=0.496)	-0.07 (-0.35 to 0.21, p=0.621)	-0.00 (-0.47 to 0.47, p=0.993)
Abacavir	0.07 (-0.18 to 0.31, p=0.593)	0.11 (-0.16 to 0.39, p=0.419)	0.09 (-0.19 to 0.36, p=0.543)	NA
LagTime				
Smoking (pckyrs)	0.00 (-0.00 to 0.01, p=0.055)	0.00 (-0.00 to 0.01, p=0.052)	0.00 (-0.00 to 0.01, p=0.104)	0.00 (-0.00 to 0.01, p=0.100)
HIV duration	0.01 (-0.01 to 0.03, p=0.274)	0.00 (-0.02 to 0.03, p=0.739)	0.01 (-0.01 to 0.03, p=0.533)	0.01 (-0.02 to 0.03, p=0.539)
cART duration	-0.01 (-0.04 to 0.01, p=0.309)	-0.01 (-0.04 to 0.02, p=0.354)	-0.02 (-0.04 to 0.01, p=0.270)	-0.02 (-0.04 to 0.01, p=0.281)
HIV RNA zenith	0.00 (-0.00 to 0.01, p=0.135)	0.01 (-0.00 to 0.01, p=0.109)	0.01 (-0.00 to 0.01, p=0.115)	0.01 (-0.00 to 0.01, p=0.113)
CD4 nadir	0.02 (-0.07 to 0.11, p=0.611)	-0.00 (-0.11 to 0.10, p=0.951)	0.02 (-0.08 to 0.12, p=0.702)	0.02 (-0.08 to 0.13, p=0.665)
CD4 count	-0.07 (-0.14 to 0.00, p=0.065)	-0.07 (-0.16 to 0.01, p=0.078)	-0.07 (-0.15 to 0.01, p=0.106)	-0.07 (-0.15 to 0.01, p=0.104)
CD4-CD8 ratio	-0.05 (-0.09 to -0.01, p=0.015)	-0.06 (-0.10 to -0.02, p=0.007)	-0.06 (-0.10 to -0.02, p=0.006)	-0.06 (-0.10 to -0.02, p=0.006)
CD4 recovery (relative)	-0.24 (-0.43 to -0.06, p=0.009)	-0.30 (-0.50 to -0.10, p=0.004)	-0.25 (-0.45 to -0.05, p=0.013)	-0.25 (-0.45 to -0.05, p=0.014)
INSTI	0.08 (-0.08 to 0.23, p=0.319)	0.15 (-0.02 to 0.33, p=0.079)	0.19 (0.03 to 0.36, p=0.024)	0.19 (0.03 to 0.36, p=0.025)
Raltegravir	0.13 (-0.06 to 0.32, p=0.175)	0.22 (0.01 to 0.44, p=0.044)	0.28 (0.07 to 0.49, p=0.008)	0.29 (0.08 to 0.51, p=0.007)
Elvitegravir	-0.38 (-0.65 to -0.11, p=0.006)	-0.34 (-0.64 to -0.05, p=0.023)	-0.31 (-0.60 to -0.03, p=0.033)	-0.33 (-0.63 to -0.03, p=0.031)
Dolutegravir	0.09 (-0.05 to 0.24, p=0.213)	0.11 (-0.05 to 0.27, p=0.187)	0.10 (-0.06 to 0.26, p=0.220)	0.11 (-0.06 to 0.29, p=0.213)
PI	0.21 (0.02 to 0.41, p=0.033)	0.19 (-0.04 to 0.42, p=0.111)	0.20 (-0.02 to 0.42, p=0.080)	0.21 (-0.02 to 0.44, p=0.070)
NNRTI	-0.13 (-0.29 to 0.02, p=0.095)	-0.19 (-0.36 to -0.01, p=0.038)	-0.23 (-0.40 to -0.07, p=0.007)	-0.24 (-0.41 to -0.07, p=0.007)
Tenofovir (TDF)	-0.04 (-0.19 to 0.10, p=0.568)	-0.02 (-0.19 to 0.14, p=0.764)	-0.00 (-0.16 to 0.15, p=0.950)	0.03 (-0.22 to 0.28, p=0.840)
Lamivudine	0.02 (-0.12 to 0.16, p=0.782)	0.01 (-0.15 to 0.17, p=0.890)	0.01 (-0.15 to 0.16, p=0.922)	-0.03 (-0.31 to 0.25, p=0.853)
Emtricitabine	-0.08 (-0.22 to 0.07, p=0.283)	-0.07 (-0.23 to 0.09, p=0.418)	-0.04 (-0.20 to 0.11, p=0.591)	-0.08 (-0.34 to 0.19, p=0.563)
Abacavir	0.03 (-0.11 to 0.18, p=0.668)	0.04 (-0.12 to 0.21, p=0.586)	0.02 (-0.14 to 0.17, p=0.810)	NA

nETPTMsr				
Smoking (pckyrs)	-0.00 (-0.01 to -0.00, p=0.013)	-0.00 (-0.01 to -0.00, p=0.019)	-0.00 (-0.01 to -0.00, p=0.021)	-0.00 (-0.01 to -0.00, p=0.018)
HIV duration	-0.02 (-0.04 to -0.00, p=0.028)	-0.02 (-0.04 to 0.00, p=0.063)	-0.02 (-0.04 to 0.00, p=0.055)	-0.02 (-0.04 to 0.00, p=0.064)
cART duration	0.01 (-0.01 to 0.04, p=0.318)	0.01 (-0.02 to 0.04, p=0.501)	0.01 (-0.02 to 0.04, p=0.518)	0.01 (-0.02 to 0.04, p=0.561)
HIV RNA zenith	-0.01 (-0.01 to -0.00, p=0.006)	-0.01 (-0.01 to -0.00, p=0.017)	-0.01 (-0.01 to -0.00, p=0.017)	-0.01 (-0.01 to -0.00, p=0.016)
CD4 nadir	-0.01 (-0.08 to 0.07, p=0.887)	-0.00 (-0.09 to 0.08, p=0.915)	-0.01 (-0.09 to 0.08, p=0.823)	-0.02 (-0.10 to 0.07, p=0.691)
CD4 count	0.10 (0.04 to 0.16, p=0.002)	0.10 (0.03 to 0.16, p=0.005)	0.10 (0.03 to 0.17, p=0.004)	0.10 (0.03 to 0.17, p=0.003)
CD4-CD8 ratio	-0.02 (-0.05 to 0.02, p=0.355)	-0.01 (-0.05 to 0.03, p=0.633)	-0.01 (-0.05 to 0.03, p=0.639)	-0.01 (-0.05 to 0.03, p=0.594)
CD4 recovery (relative)	0.10 (-0.07 to 0.27, p=0.236)	0.09 (-0.09 to 0.26, p=0.321)	0.09 (-0.09 to 0.27, p=0.300)	0.09 (-0.09 to 0.27, p=0.315)
INSTI	-0.08 (-0.22 to 0.05, p=0.234)	-0.12 (-0.26 to 0.02, p=0.102)	-0.12 (-0.26 to 0.03, p=0.106)	-0.11 (-0.25 to 0.03, p=0.131)
Raltegravir	-0.10 (-0.27 to 0.08, p=0.267)	-0.17 (-0.35 to 0.01, p=0.061)	-0.17 (-0.36 to 0.01, p=0.060)	-0.19 (-0.37 to -0.00, p=0.045)
Elvitegravir	0.22 (-0.00 to 0.45, p=0.054)	0.19 (-0.04 to 0.42, p=0.097)	0.19 (-0.04 to 0.42, p=0.104)	0.18 (-0.07 to 0.42, p=0.153)
Dolutegravir	-0.08 (-0.20 to 0.05, p=0.234)	-0.07 (-0.20 to 0.07, p=0.331)	-0.06 (-0.20 to 0.07, p=0.354)	-0.05 (-0.20 to 0.10, p=0.531)
PI	-0.09 (-0.26 to 0.09, p=0.321)	-0.10 (-0.28 to 0.08, p=0.271)	-0.10 (-0.29 to 0.08, p=0.268)	-0.12 (-0.31 to 0.07, p=0.205)
NNRTI	0.09 (-0.05 to 0.23, p=0.193)	0.13 (-0.01 to 0.28, p=0.069)	0.14 (-0.01 to 0.28, p=0.070)	0.13 (-0.02 to 0.28, p=0.093)
Tenofovir (TDF)	0.08 (-0.05 to 0.20, p=0.233)	0.06 (-0.07 to 0.19, p=0.336)	0.06 (-0.07 to 0.19, p=0.359)	0.04 (-0.16 to 0.25, p=0.667)
Lamivudine	-0.05 (-0.18 to 0.08, p=0.424)	-0.05 (-0.18 to 0.08, p=0.478)	-0.04 (-0.17 to 0.09, p=0.509)	0.01 (-0.22 to 0.24, p=0.959)
Emtricitabine	0.04 (-0.08 to 0.17, p=0.501)	0.03 (-0.10 to 0.16, p=0.691)	0.02 (-0.11 to 0.16, p=0.726)	-0.05 (-0.27 to 0.16, p=0.618)
Abacavir	-0.07 (-0.19 to 0.06, p=0.307)	-0.06 (-0.19 to 0.07, p=0.372)	-0.06 (-0.19 to 0.08, p=0.402)	NA

Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (nETPTMsr).Linear regression was used for analysis. Data shown as Beta with confidence interval and p-value. Model 1 includes age, time of inclusion and sex as covariates. Model 2 also includes sCD14 as a covariate. Model 3 includes abacavir use as covariate.

Supplementary Table 6. Subgroup analysis restricting to PLHIV on a non-abacavir (ABC) containing regimen and healthy controls only.

	PLHIV-no ABC (n=114)	HC (n=56)	p
AGE (mean (SD))	52.3 (10.3)	39.9 (17.3)	<0.001
BMI (median [IQR])	24.2 [22.4, 26.0]	23.8 [21.5, 25.6]	0.395
Sex (%female)	13 (11.4)	22 (39.3)	<0.001
iFABP (median [IQR])	576.2 [287.4, 897.2]	242.5 [112.9, 376.9]	<0.001#
hsCRP (median [IQR])	1558 [624, 3285]	651.2 [205.9, 1179.2]	<0.001#
sCD14 (median [IQR])	2063 [1745, 2591]	1789 [1502, 2071]	0.001#
sCD163 (median [IQR])	765.8 [581.6, 916.1]	517.3 [410.7, 578.1]	<0.001#
Plasma vWf (median [IQR])	42344.2 [31562.4, 61642.5]	32468.9 [2441.6, 43935.1]	<0.001#
d.dimer (median [IQR])	213.4 [158.6, 335.5]	170.5 [122.0, 307.2]	0.01#
Fibrinogen (median [IQR])	3.4 [2.8, 4.0]	3.2 [2.8, 4.1]	0.589
Prothrombin (median [IQR])	106.0 [87.1, 135.1]	135.1 [102.4, 162.5]	<0.001#
ProteinS (median [IQR])	91.7 [81.7, 109.4]	86.9 [72.7, 112.4]	0.163
ETP (median [IQR])	83.7 [74.7, 97.6]	93.0 [83.3, 105.0]	<0.001#
LagT (median [IQR])	2.0 [1.7, 2.3]	2.0 [1.7, 2.0]	0.618
TTP (median [IQR])	4.3 [4.0, 5.0]	4.3 [3.5, 5.0]	0.103
Peak (median [IQR])	79.9 [65.2, 96.0]	88.1 [72.3, 101.5]	0.012
VI (median [IQR])	76.4 [53.6, 102.5]	97.3 [64.0, 123.5]	0.002
LagT_TM (median [IQR])	1.7 [1.4, 2.3]	1.7 [1.4, 2.0]	0.996
TTP_TM (median [IQR])	3.7 [3.3, 4.0]	3.7 [3.3, 4.0]	0.687
ETP_TM (median [IQR])	41.0 [28.0, 51.1]	50.1 [32.8, 76.2]	0.003
PEAK_TM (median [IQR])	50.9 [33.2, 61.6]	57.1 [39.8, 82.5]	0.011
VI_TM (median [IQR])	62.1 [39.8, 85.4]	71.9 [51.8, 112.5]	0.004
ETP_TM_NSR (median [IQR])	0.9 [0.7, 1.2]	1.2 [0.8, 1.5]	0.012
PEAK_TM_NSR (median [IQR])	1.0 [0.8, 1.1]	1.1 [0.9, 1.3]	0.003

Markers of inflammation: High sensitive CRP (hsCRP), soluble CD14, soluble CD163. Marker of micriobial translocation: plasma IFABP. Marker of endothelial activation: plasma von willebrand factor (vWf). Calibrated thrombin generation was triggered with 5 pM tissue factor, 4 µM phospholipids and in the presence and absence of 7 nM thrombomodulin (TM). Lag time (LagT), Time to peak (TTP), endogenous thrombin production (ETP). Normalized Protein C activity (NSR). Circulating factors and ETP as the primary outcome for TG were corrected for multiple testing using Benjamini-Hochberg procedure (FDR). All remaining TG parameters were treated as exploratory parameters # Significantly different between groups after FDR-correction.

Chapter 6

Long-term treated HIV-infection is associated with platelet Mitochondrial dysfunction

Wouter van der Heijden, Lisa Van de Wijer, Martin Jaeger, Karin Grintjes, Mihai Netea, Rolf Urbanus, Reinout van Crevel, Lambertus van den Heuvel, Maaike Brink, Richard Rodenburg, Philip de Groot, Andre van der Ven, Quirijn de Mast

Scientific reports 2021; 11(1): 6246.

Abstract

HIV infection and antiretroviral therapy have been linked to mitochondrial dysfunction. The role of platelet mitochondrial dysfunction in thrombosis, immunoregulation and age-related diseases is increasingly appreciated. Here, we studied platelet mitochondrial DNA content (mtDNA $_{pl}$) and mitochondrial function in people living with HIV (PLHIV) and related this to platelet function. In a cohort of 208 treated PLHIV and 56 uninfected controls, mtDNA$_{pl}$ was quantified, as well as platelet activation, platelet agonist-induced reactivity and inflammation by circulating factors and flow cytometry. In a subgroup of participants, the metabolic activity of platelets was further studied by mitochondrial function tests and the Seahorse Flux Analyzer. PLHIV had significantly lower mtDNA$_{pl}$ compared to controls (8.5 copies/platelet (IQR:7.0-10.7) vs. 12.2 copies/platelet (IQR:9.5-16.6); $p < 0.001$), also after correction for age, sex and BMI. Prior zidovudine-use (n=46) was associated with a trend for lower mtDNA$_{pl}$. PLHIV also had reduced *ex vivo* platelet reactivity and mean platelet volume compared to controls. MtDNA$_{pl}$ correlated positively with both platelet parameters and correlated negatively with inflammatory marker sCD163. Mitochondrial function tests in a subgroup of participants confirmed the presence of platelet mitochondrial respiration defects. Platelet mitochondrial function is disturbed in PLHIV, which may contribute to platelet dysfunction and subsequent complications. Interventions targeting the preservation of normal platelet mitochondrial function may ultimately prove beneficial for PLHIV.

Background

Mitochondrial dysfunction is a well-known phenomenon in people living with HIV (PLHIV), which has been linked with the use of nucleoside reverse transcriptase inhibitors (NRTIs)[1-5]. The main mechanism underlying NRTI toxicity is inhibition of mitochondrial DNA polymerase γ and increased oxidative stress, resulting in mitochondrial DNA (mtDNA) depletion[6-8], and mitochondrial dysfunction at the tissue level[7,9]. These adverse effects were greatly reduced when the older NRTIs stavudine, zidovudine (AZT) and didanosine were replaced by the newer NRTIs tenofovir (TDF) and abacavir (ABC). However, these newer NRTIs may still impair mitochondrial function, albeit to a lesser degree[10-13]. More recently, mitochondrial dysfunction has also been reported in people living with HIV (PLHIV) naive for combined antiretroviral therapy (cART)[3,14-16], including in elite-controllers [17]. The factors responsible for cART-independent mtDNA depletion are less well defined and may involve persistent immune activation[18,19]. Mitochondrial dysfunction has been suggested to contribute to non-AIDS related co-morbidities such as cardiovascular diseases, diabetes, cancer and dementia in PLHIV[20,21].

Platelets are the second most numerous blood cells that are, unlike red blood cells, equipped with mitochondria with mtDNA[22,23]. Human platelets lack a nucleus and mitochondria are essential in maintaining platelet health and lifespan, as recently reviewed[23]. MtDNA copy number is considered to reflect mitochondrial function[24]. Healthy platelets contain between 5 and 8 mitochondria, which serve important processes such as platelet activation, ATP production and platelet viability[25-29]. Platelets are traditionally known for their role in hemostasis, but an increasing body of evidence supports their role in key processes beyond hemostasis, including inflammation and immunoregulation[23]. With the importance of mitochondria in platelet metabolism, it is no surprise that the health consequences of abnormalities in platelet mitochondrial DNA and function has received increased attention, and platelet mtDNA has been proposed to serve as biomarker for different diseases[22,30-32]. Data on platelet function in PLHIV is contradictory, with some studies reporting increased [33-37], but other reduced agonist-induced platelet reactivity[28,38,39].

We hypothesized that mitochondrial dysfunction in PLHIV is associated with reduced platelet mtDNA copies and platelet dysfunction. Here, we show that platelet mtDNA copies are lower in PLHIV on long-term cART and this was validated in a subgroup of PLHIV using mitochondrial functional assays.

Results

Cohort characteristics

Between December 2016 and February 2017, a total of 208 virally suppressed PLHIV on long-term cART and 56 healthy controls (sampled twice) were concurrently enrolled. Baseline characteristics are shown in Table 1. PLHIV were older compared to controls (52 years (IQR: 45.8-59.0) vs 30 years (IQR: 25.8-53), respectively, $p < 0.001$). Median duration of cART use was 6.6 yrs (IQR: 4.2-11.9).

Platelet mtDNA copies are reduced in PLHIV

Platelet mtDNA copies ($mtDNA_{pl}$) were significantly lower in PLHIV compared to healthy controls (Figure 1A; median 8.5 copies/platelet (plt) (IQR 7.0-10.7) vs 12.2 copies/plt (IQR: 9.5-16.6), p < 0.001). $mtDNA_{pl}$ copies correlated inversely with age (Figure 1B; Pearson's R=-0.24, p < 0.001). $MtDNA_{pl}$ remained significantly associated with HIV status after correction for age, sex and body-mass index (BMI) in a linear regression model (B=0.77 SE:0.14, P<0.0001). When analysis was restricted to individuals of 40 years and above (Supplemental Table S1; PLHIV: n=173 vs HC: n=22), $mtDNA_{pl}$ remained significantly lower in PLHIV compared to controls (Supplemental Figure 1; PLHIV: 8.4 copies/plt (IQR: 6.6-10.2) vs HC: 10.5 copies/plt (IQR:8.9-14.7), p = 0.001). Similarly, the differences in $mtDNA_{pl}$ between PLHIV and controls remained when analysis was restricted to males only (Supplemental Table S2; PLHIV: 8.5 copies/plt (IQR: 6.6-10.7) vs HC: 11.6 copies/plt (IQR:9.3-14.7), p < 0.001). $MtDNA_{pl}$ levels were neither associated with duration of cART nor with current CD4 count, CD4 nadir or CD4/CD8 ratio in univariate analysis (Supplemental Table S3). Furthermore, no differences in $mtDNA_{pl}$ were found between PI, NNRTI and INSTI-based regimens (all p > 0.1; Supplemental Figure S2).

Although abacavir (ABC)-use has been shown to affect platelet function[40], current ABC use ($p = 0.89$) or cumulative ABC exposure (in days) were not associated with $mtDNA_{pl}$ copies (Supplemental Figure S2). Conversely, PLHIV with prior zidovudine-use (prior AZT-use, n=46/184; 25%) showed a trend towards lower copies of $mtDNA_{pl}$ (AZT-use: 7.8 copies/plt [IQR:6.0, 9.4] vs never-AZT: 8.6 copies/plt [IQR: 6.9, 10.8], $p = 0.055$). After correcting for age in a linear regression model, a non-significant negative correlation remained (B:-0.07 (-0.15 to 0.01, p = 0.069). Neither cumulative days on AZT (median 2069 days; IQR 792-3180 days; R=-0.2, $p = 0.12$), nor total NRTI exposure (median 4520 days; IQR: 2734-7955; R = 0.05) correlated with mtDNA-use. Use of metformin (p = 0.079) or antihypertensive drugs (p = 0.061) showed a tendency for a lower $mtDNA_{pl}$ copies, whereas use of statins ($p = 0.74$) or acetylsalicylic acid ($p = 0.21$) did not (Supplemental Figure S3).

Table 1. Baseline characteristics

Characteristics	PLHIV (n=208)	Healthy controls (n=56)
Sex (% Female)	17 (8.2)	22 (39.2)[a]
Age (years, median [IQR])	52.0 [45.8, 59.0]	30.0 [25.8-53.0][b]
BMI (median [IQR])	24.1 [22.0, 26.0]	23.8 [21.5-25.6]
HIV infection duration (years, median [IQR])	8.5 [5.0, 14.2]	
Way of transmission (%)		
Heterosexual	8 (3.8)	
IDU	3 (1.4)	
MSM	158 (76.0)	
Other/unknown	39 (18.8)	
CD4 nadir (median [IQR])	250.0 [135.0, 362.5]	
CD4 count (median [IQR])	660.0 [480.0, 812.5]	
Undetectable HIV load, n (%)	208 (100)	
CD4/CD8 ratio (median [IQR])	0.8 [0.6, 1.1]	
cART duration (years; median [IQR])	6.6 [4.1, 11.8]	
cART regimen		
NRTI-use (%)	200 (96.2)	
NtRTI-use (%)	97 (46.6)	
NNRTI-use (%)	61 (29.3)	
PI-use (%)	32 (15.4)	
Maraviroc-use (%)	3 (1.4)	
INSTI-use (%)	140 (67.3)	
ABC (%)	93 (44.7)	
DTG (%)	86 (41.3)	
EVG (%)	15 (7.2)	
RAL (%)	38 (18.3)	
Smoking (%)	59 (28.4)	
Pack years (median [IQR])	13.8 [0.0, 28.0]	
Hypercholesterolemia (%)	56 (26.9)	
Hypertension (%)	40 (19.2)	
Diabetes Mellitus (%)	9 (4.3)	
No cardiovascular risk factors (%)	50 (24.0)	
Statins (%)	56 (26.9)	
Aspirin (%)	18 (8.7)	
Metformin (%)	9 (4.3)	

BMI: body mass index. cART: combination antiretroviral therapy. NRTI: Nucleoside reverse transcriptase inhibitor. NRTI: nucleotide reverse transcriptase inhibitor. NNRTI: non-nucleoside reverse transcriptase inhibitor. PI: protease inhibitor. INSTI: integrase inhibitor. ABC: abacavir. DTG: dolutegravir. EVG: elvitegravir. RAL: raltegravir. *Significantly different between cohorts

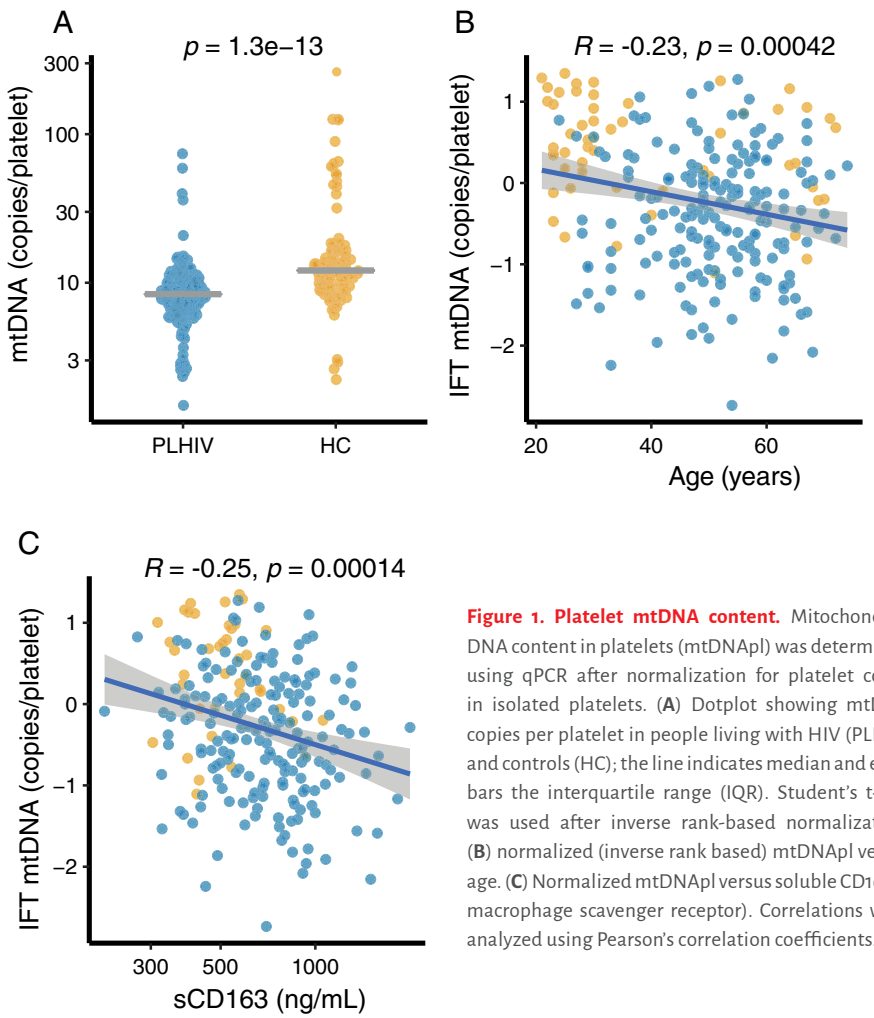

Figure 1. Platelet mtDNA content. Mitochondrial DNA content in platelets (mtDNApl) was determined using qPCR after normalization for platelet count in isolated platelets. (**A**) Dotplot showing mtDNA copies per platelet in people living with HIV (PLHIV) and controls (HC); the line indicates median and error bars the interquartile range (IQR). Student's t-test was used after inverse rank-based normalization. (**B**) normalized (inverse rank based) mtDNApl versus age. (**C**) Normalized mtDNApl versus soluble CD163 (a macrophage scavenger receptor). Correlations were analyzed using Pearson's correlation coefficients.

Mitochondrial dysfunction is associated with inflammatory diseases such as atherosclerosis and sepsis in HIV-negative individuals [22]. Hence, we explored the association of known markers of persisting immune activation, a known driver of non-AIDS related comorbidities[20,21], with $mtDNA_{pl}$ copy number. Overall, we observed higher plasma concentrations of immune activation markers soluble CD14 (sCD14), soluble CD163 (sCD163) and high-sensitive CRP (hsCRP) in PLHIV than in controls (Table 2). Among participants older than 40 years, only sCD163 remained significantly different between groups (Supplemental table S1; HIV 741 ng/mL (IQR: 547-906) vs controls 517 ng/mL (IQR: 443-684), p = 0.01). sCD163 correlated negatively with $mtDNA_{pl}$ copies (R = -0.23, p < 0.001, Figure 1c), whereas sCD14 (R = -0.073, p = 0.27) and hsCRP (R: -0.087, p = 0.2) did not (Supplemental Figure S4).

Platelet mitochondrial dysfunction in PLHIV

Energy demand for platelet ATP production and other metabolic processes that are essential for platelet activation is met by the combined actions of glycolysis and mitochondrial OXPHOS[42]. To validate our findings that the lower platelet mtDNA content is associated with platelet mitochondrial dysfunction, and to investigate platelet glycolysis activity, we assessed the metabolic activity of washed platelets of five PLHIV and five age-sex matched controls to assess membrane potential ($\Delta\psi_m$), mitochondrial superoxide production (ROS_m) and real-time glycolysis and mitochondrial respiration using the Seahorse Extracellular Flux Analyzer[42]. Platelet $\Delta\psi_m$, as assessed by Tetramethylrhodamine ethyl ester (TMRE) fluorescence, was lower in PLHIV compared to controls (1669 ΔMFI (IQR: 911-2233) vs. 4545 ΔMFI (IQR: 2249-5168) respectively, p = 0.02; figure 2e, t-test). In line with reduced $\Delta\psi_m$, there was a small increase in ROS_m in PLHIV at basal conditions (figure 2f; 7654 MFI (IQR: 6777-7798) vs 4956 MFI (IQR: 3840-6284), p = 0.007, t-test). Next, using the Seahorse Extracellular Flux Analyzer, mitochondrial respiration and glycolytic capacity were investigated by assessing the oxygen consumption rate (OCR; measure for mitochondrial respiration/OXPHOS) and the acidification rate (ECAR; measure for glycolysis). There was a trend towards lower baseline platelet mitochondrial respiration (OCR in pmol/min) in PLHIV compared to controls (Figure 2d; 47.4 pmol/min (SD: 11.3) vs 55.1 pmol/min (SD: 12.2), p = 0.084). Additionally, PLHIV had a smaller increase in OCR after *ex vivo* platelet stimulation with both CRP-XL (108% (SD: 16) vs 119% (SD: 16), p = 0.056, Figure 2b and Supplemental Figure 5) and Thrombin receptor activating peptide (TRAP; 139% (SD: 14) vs 167% (SD: 13), p = 0.037; Figure 2a-c) compared to matched controls.

The fraction of the maximal mitochondrial capacity (maximal OCR after stimulation with the uncoupling agent FCCP) used after *ex vivo* platelet stimulation with TRAP was high in both groups (Supplemental Figure S6d; p > 0.1). This suggests that maximal mitochondrial capacity is a limiting factor in platelet activation. Conversely, there was no difference between groups in ECAR (glycolysis) at baseline (Figure 2b; HIV: 16.0 mpH/min (SD: 3.4) vs HC: 14.7mpH/min (SD: 2.4), p > 0.1) or after *ex vivo* stimulation. The increase in mitochondrial respiration (OCR) after *ex vivo* stimulation significantly correlated with $mtDNA_{pl}$ (R=0.77, p = 0.045, n=7, Figure 3a) and a similar trend was found for basal mitochondrial respiration of platelets (R=0.61, p = 0.14, n=7, Figure 3b). Taken together, our data suggest that the lower platelet mtDNA in PLHIV in associated with a concurrent reduction in mitochondrial respiration capacity (OXPHOS) without a compensatory increase in glycolysis.

HIV infection is associated with platelet dysfunction

Next, we explored the possible consequences of lower $mtDNA_{pl}$ and mitochondrial dysfunction for platelet parameters and function. Platelet counts were similar between PLHIV and healthy controls (Table 2), but PLHIV had smaller platelets (mean platelet volume: PLHIV:10.1 fL (IQR: 9.7:10.7) vs 10.8 fL (IQR: 10.3-11.3), $p < 0.0001$), as well as a lower immature platelet fraction (IPF; a marker for freshly released platelets from the bone marrow) compared to controls (Table 2; 3.3% (IQR: 2.5-4.6) vs 3.7% (IQR: 2.8-5.9) respectively, $p = 0.018$). Platelet size (mean platelet volume) correlated positively with $mtDNA_{pl}$ (Figure 5c).

Next, platelet activation and function and function were determined using multiple methods. First, plasma markers of *in vivo* platelet activation (chemokines released from alpha-granules; CCL5, CXCL4, CXCL7) were comparable between PLHIV and controls (Table 2). Second, using flow cytometry, the activation status of circulating platelets, as well as their reactivity to *ex vivo* stimulation by platelet agonists was assessed. In unstimulated platelets, the expression of the alpha-granule marker P-selectin (measure of platelet degranulation) and the binding of fibrinogen to the activated integrin αIIbβ3 (measure of aggregation; Table 2) were also similar across groups. When analysis was restricted to individuals above 40 years of age or male only, unstimulated platelet activation was lower in PLHIV compared to controls (Supplemental Table S1-2). In line with this observation, fibrinogen binding to αIIbβ3 in response to stimulation by adenosine diphosphate (ADP)- and collagen related peptide (CRP-XL) was reduced in PLHIV-induced) (Figure 4a). Differences in P-selectin reactivity across the groups were smaller with only a significant difference with a high dose (125μM) of ADP stimulation showed a significant difference between PLHIV and controls (Figure 4b). We observed no correlations with cART regimens containing either NNRTI, PI or INSTI-use as well as ABC and platelet reactivity indices (all $P>0.15$). In addition, persistent immune activation did not correlate with platelet indices in this cohort (Supplemental Table S4). In summary, these data show that platelet reactivity, and especially αIIbβ3 activation is reduced in PLHIV.

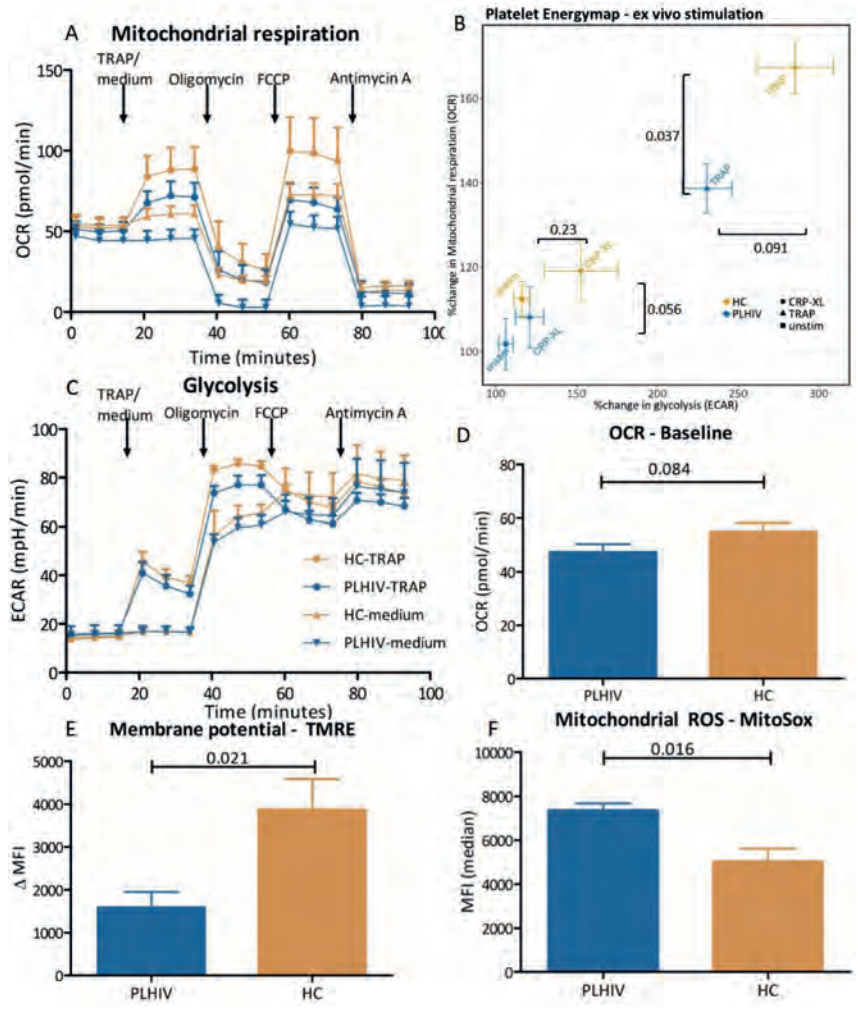

Figure 2. Platelet energy phenotype determined by the Seahorse extracellular flux analyzer and mitochondrial membrane potential and ROS production. Samples were measured with five replicates, 1.10^7 washed platelets per well. Five age-and sex-matched donors per group. (**A**) Real-time mitochondrial respiration depicted as oxygen consumption rate (OCR) at 18 timepoints. Four agonists or inhibitors were added in the following order: 1) Thrombin Receptor Activator Peptide-6 (TRAP-6; 50μM) or medium causing platelet activation; 2) Oligomycin which inhibits cellular ATP production; 3) Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), an uncoupling agent causing maximum oxygen consumption through complex IV, and 4) Antimycin A which inhibits all mitochondrial respiration (CRP-XL can be found in supplemental figure 9). (**B**) Energymap showing the change in mitochondrial respiration (as change in OCR) and glycolysis (extracellular acidification rate (ECAR) from basal conditions after ex vivo stimulation with platelet agonists. (**C**) Depicts the real-time ECAR after 1) medium or TRAP-6 2) oligomycine, 3) FCCP and 4) antimycin A. Samples were measured with five replicates, 1.10^7 washed platelets per well. Five age-and sex-matched donors per group. (**D**) Basal OCR as mean of first three measurements. (**E**) Membrane potential was determined using Tetramethylrhodamine ethyl ester (TMRE) staining with the uncoupler FCCP disrupting mitochondrial membrane potential as a negative control for every sample. Data depicted as delta geometric mean fluorescence intensity (MFI). (**F**) Mitochondrial ROS production with MitoSox staining in MFI. Data are depicted as mean ±standard error of the mean (SEM) and analyzed using the unpaired student's T-test.

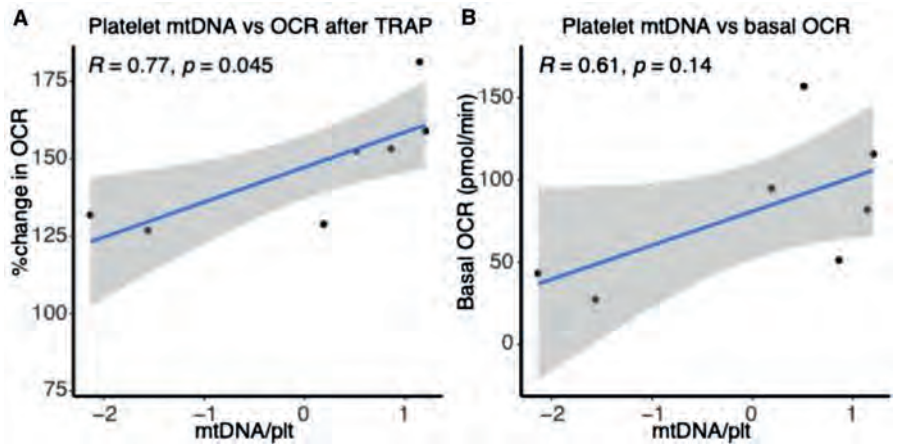

Figure 3. Platelet mitochondrial DNA vs mitochondrial respiration measured by Oxygen consumption rate (OCR in pmol/min). (**A**) Correlation plot showing platelet mitochondrial DNA (mtDNA) with change in oxygen consumption rate (mitochondrial respiration) measured by Seahorse extracellular flux analyzer after ex vivo stimulation with thrombin (TRAP 50uM). mtDNA was normalized using inverse rank-based transformation and Pearson's correlation coefficient is shown. (**B**) mtDNA in platelets vs basal OCR in pmol/min (an average of first three measurements). OCR was measured in triplo and for 5 PLHIV and 2 controls both mtDNA in platelets and real-time measurements of mitochondrial respiration were available and included in the analysis.

Table 2

Platelet indices	PLHIV (n=208)	Healthy controls (n=56)	p -value
Platelet count (10^9/L)	260 [200, 310]	270 [210, 320]	0.126
MPV (fL)	10.1 [9.7, 10.7]	10.8 [10.3, 11.3]	<0.001
IPF (%)	3.3 [2.5, 4.6]	3.7 [2.8, 5.9]	0.018
Unstimulated fibrinogen binding (MFI)	2.0 [1.6, 2.2]	2.1 [1.6, 2.4]	0.072
Unstimulated P-selectin expression (MFI)	2.7 [2.1, 3.2]	2.6 [2.2, 3.0]	0.158
Plasma CCL5 (ng/ml)	2.66 [1.64, 4.29]	2.61 [1.69, 4.18]	0.841
Plasma CXCL4 (ng/ml)	570.0 [290.0, 723.3]	492.9 [306.1, 839.5]	0.201
Plasma CXCL7 (ng/ml)	289.4 [180.3, 465.1]	306.6 [194.2, 609.8]	0.192
sCD14 (ng/ml)	2139.6 [1778.2, 2661.5]	1789.0 [1502.7, 2071.6]	<0.001
sCD163 (ng/ml)	716.5 [528.7, 899.4]	517.3 [410.7, 578.1]	<0.001
hsCRP (ng/ml)	1423.3 [608.8, 2726.3]	651.2 [205.9, 1179.2]	<0.001

MPV: mean platelet volume. IPF: immature platelet fraction as a percentage of platelet count (Sysmex, Kobe, Japan). unstimulated platelet aggregation measured as Fibrinogen binding by flowcytometry in median fluorescence intensity (MFI). Unstimulated platelet degranulation measured as P-selectin expression by flow cytometry by MFI. sCD14: Serum levels of CD14, a marker of monocyte activation. sCD163: Serum levels of CD163, a monocyte- and macrophage-specific scavenger receptor. hsCRP: high sensitive C-reactive protein

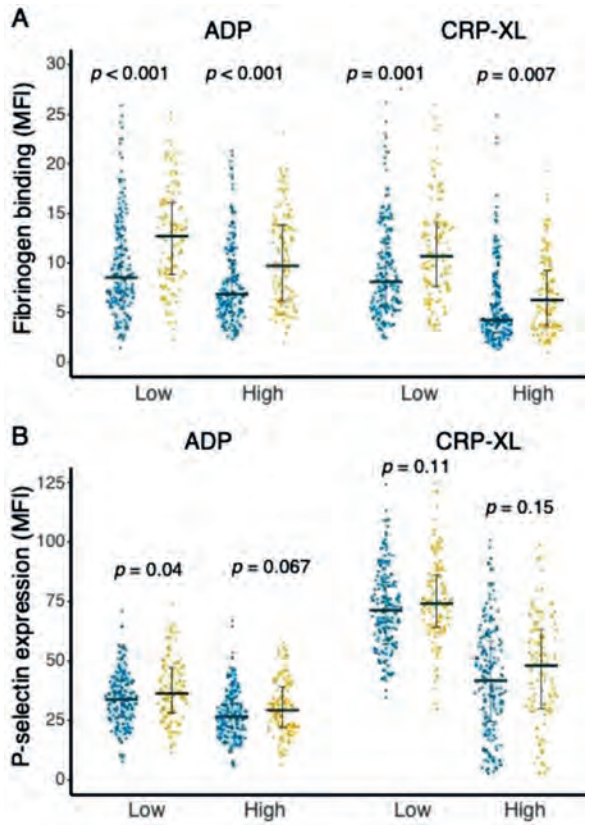

Figure 4. Platelet reactivity after collagen (CRP-XL) and ADP stimulation. (**A**) Platelet fibrinogen binding is depicted as mean fluorescence intensity (MFI) after ex vivo stimulation with platelet agonist collagen-related peptide (CRP-XL; 33ng/mL; 625ng/mL) and adenosine diphosphate (ADP; 1.2uM, 125uM). (**B**) Platelet degranulation measured by P-selectin expression is depicted as mean fluorescence intensity (MFI) after *ex vivo* stimulation with CRP-XL (33ng/mL; 625ng/mL) and ADP (1.2uM, 125uM). Data are shown as dotplot with error bars median and interquartile range (IQR). Data were analyzed using unpaired Student's T-test. People living with HIV (PLHIV); healthy controls (HC).

Association of $mtDNA_{pl}$ with platelet function

Next, given the role of platelet mitochondria in platelet function, we assessed associations of $mtDNA_{pl}$ with platelet reactivity in PLHIV and controls. $MtDNA_{pl}$ copies did neither correlate with ADP-induced P-selectin expression, nor with binding of fibrinogen to platelets or mean platelet volume (MPV) in PLHIV (Figure 5a-b). In controls, $MtDNA_{pl}$ copies were significantly correlated with MPV (Figure 5c) and a positive trend was observed with fibrinogen binding (Figure 5d).

To further explore the link between platelet reactivity and platelet activation, we performed a principal component analysis (PCA) to summarize both platelet activation (plasma markers of platelet activation and unstimulated P-selectin expression and fibrinogen binding) and platelet reactivity (P-selectin expression and fibrinogen binding after *ex vivo* ADP and CRP-XL stimulation). This PCA showed that Principal component (PC) 1 mainly represented platelet reactivity (P-selectin expression and fibrinogen binding after stimulation) whereas PC2 mainly represented *in vivo* platelet activation (plasma markers and unstimulated

P-selectin expression and fibrinogen binding; Supplemental Figure S7a-b). We used these derivatives to correlate $mtDNA_{pl}$ with platelet reactivity (coordinates on PC1) and platelet activation (coordinates on PC2). $mtDNA_{pl}$ correlated with platelet reactivity (Supplemental Figure S7c; PC1 of platelet parameters vs $mtDNA_{pl}$, R=0.14, p=P=0.024), but not with *in vivo* platelet activation (Supplemental Figure S7d; PC2 of platelet parameters vs $mtDNA_{pl}$, R=0.05, P=0.41). These data suggest that mtDNA depletion is associated with platelet dysfunction with a reduced platelet reactivity capacity, but not with increased platelet activation status.

Whereas mitochondrial dysfunction can result in platelet dysfunction, platelet activation itself may also contribute to loss of mitochondria from platelets through the formation of platelet microparticles (PMP) formation.[41] We therefore studied PMP in a subgroup of 20 PLHIV and age-sex matched controls. No differences in total PMP number (Supplemental Figure S8a), nor PMPs containing mitochondria (Supplemental Figure S8b) were observed across both groups.

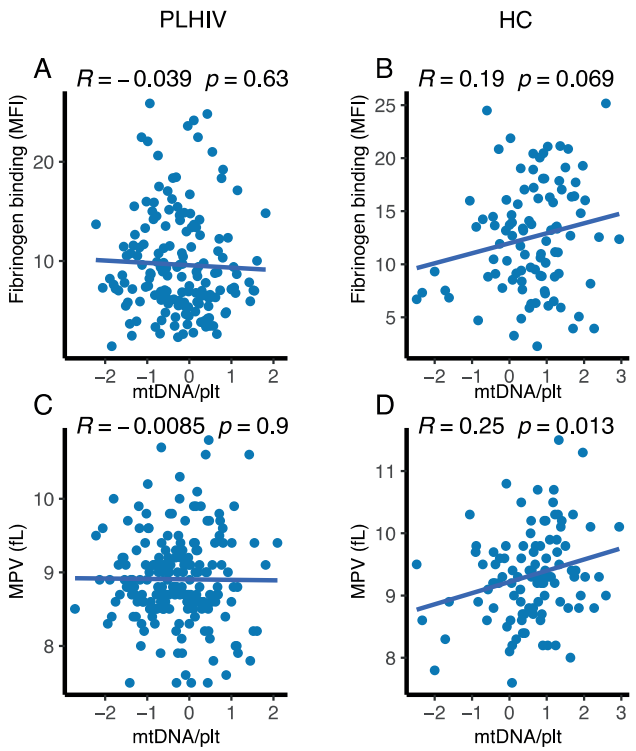

Figure 5. Association of platelet mitochondrial DNA with platelet reactivity and mean platelet volume (MPV). (**A** and **B**) correlation of normalized mitochondrial DNA content in platelets (mtDNApl) and the binding of fibrinogen to the activated integrin αIIbβ3 after ex vivo platelet stimulation with ADP (125uM) in PLHIV (**A**) and healthy controls (**B**). (**C and D**) correlation of normalized mitochondrial DNA content in platelets (mtDNApl) and MPV in PLHIV (**C**) and healthy controls (**D**). All correlations were analyzed using Pearson's correlation coefficients

Discussion

The present data show that PLHIV on long-term cART have reduced platelet mitochondrial content ($mtDNA_{pl}$) which was associated with platelet mitochondrial dysfunction and reduced energy supply. Platelet mitochondria play a key role in platelet metabolism, ATP production and platelet activation and lifespan, and we propose that the observed abnormalities in $mtDNA_{pl}$ and platelet mitochondrial function contribute to platelet dysfunction in PLHIV.

The literature on platelet function in cART treated individuals is contradictory, with some studies reporting increased platelet reactivity[33-37], while others reporting reduced reactivity.[28,38,39] This heterogeneity in study results may be partly explained by differences in the characteristics of study participants, including the enrolment of PLHIV with detectable plasma HIV-RNA, the degree of persistent immune activation, cART regimens and timing of treatment initiation, factors that all have changed considerably over the years. In accordance with our present findings, Mesquita et al. [28] recently reported decreased platelet reactivity and platelet mitochondrial dysfunction in 36 PLHIV on stable cART. In contrast to our findings, platelets in the PLHIV in their cohort exhibited increased P-selectin expression. In the present study, not only platelet P-selectin expression, but also soluble markers of platelet activation were similar in PLHIV and controls. In addition, PLHIV exhibited a lower immature platelet fraction and a similar number of mitochondria-containing platelet microparticles.

Together, these findings argue against excessive platelet activation being primarily responsible for reduced platelet reactivity in PLHIV. Whether mitochondrial depletion contributes to the observed platelet dysfunction in PLHIV remains uncertain. A recent study reported that chemotherapy-associated platelet hyporeactivity was caused by mitochondrial dysfunction and subsequent reduction in mitochondrial respiration[27]. Consistent with these observations, the lower platelet mtDNA content in PLHIV was associated with a reduction in mitochondrial respiration capacity, which may negatively impact platelet reactivity. In our study, however, $mtDNA_{pl}$ levels in PLHIV did not correlate with ex vivo platelet reactivity, whereas a positive trend was observed in HIV uninfected controls. The fact that all PLHIV exhibited reduced $mtDNA_{pl}$ with little variation in the absolute values may explain the absence of a correlation with platelet reactivity measures. Future studies focusing on platelet dysfunction should incorporate mitochondrial dysfunction to corroborate our findings.

Age, history of zidovudine (AZT)-use and innate immune activation were all associated with decreased $mtDNA_{pl}$. NRTI-use is a well-known cause of mitochondrial dysfunction and mtDNA depletion[6-8]. We found that prior AZT-use was a possible risk factor for reduced $mtDNA_{pl}$, a trend that remained after correcting for age and CD4 nadir, whereas total

duration of cART-use or duration of HIV infection were not. As platelets are short-lived, it is conceivable that mitochondrial mass is reduced during thrombopoiesis and that the known bone marrow toxicity of AZT [1] is still present even after switching to newer NRTIs such as TDF or ABC. Even though these NRTIs are known to have lower mitochondrial toxicity [1], it is unclear whether long-term treatment does not exert any cumulative reduction in $mtDNA_{pl}$ too. While ABC-use has been linked to platelet perturbations in multiple studies[43-45], others could not confirm ABC associated platelet dysfunction[35,46]. In our study, neither $mtDNA_{pl}$ content nor platelet function were associated with current or prior ABC-use. Unfortunately, we could not dissect the link between the $mtDNA_{pl}$, overall NRTI exposure and duration of HIV infection itself, as exposure to NRTIs was high in the total study group. Still, as $mtDNA_{pl}$ content was lower in PLHIV than in controls, it is plausible that both NRTIs and HIV itself exert long-term changes in mitochondrial function[2]. This possible long-term NRTI effect on mitochondrial function in platelets supports recent efforts to implement NRTI sparing regimens as viable treatment options for long-term HIV treatment[47] It would be interesting to assess $mtDNA_{pl}$ content, as well as platelet function, in PLHIV who are switched to a NRTI sparing regimen.

Mitochondrial dysfunction and depletion have been associated with many diseases such as dementia, neuropsychiatric diseases, and cardiovascular diseases[22,23,30,48]. In PLHIV, these (non-AIDS related) co-morbidities have also been linked to persistent inflammation[20,21]. In our cohort, we indeed found increased levels of hsCRP, sCD14 and sCD163, but only the latter parameter was associated with mtDNA levels in platelets. Importantly, sCD163 was shown to be independently correlated with overall mortality in PLHIV and the incidence of non-AIDS related comorbidities [49,50]. Targeting mitochondrial dysfunction and inflammation may help reduce excess mortality and morbidity that is associated with HIV infection, even when treated successfully with cART[22,27]. Reducing inflammation, besides reducing NRTI exposure, could indirectly reduce oxidative stress and mitochondrial dysfunction while treatment with ROS scavengers may also have beneficial effects in reducing non-AIDS related co-morbidities.

Multiple methods of mtDNA quantification have been used in whole blood or peripheral blood mononuclear cell (PBMC) fractions in HIV and other diseases[14,32]. However, different methods of quantification, and heterogeneity of the cell composition of whole blood or PBMCs may hamper its interpretation [32,51]. It is conceivable that platelet mtDNA content could mirror mitochondrial toxicity in other cell types as well. As a single cell-type source of mtDNA, platelet mtDNA may indeed serve as a possible biomarker for mitochondrial toxicity and non-AIDS related comorbidities associated with mitochondrial dysfunction, such as neurocognitive impairment and cardiovascular disease[22].

Our study has limitations associated with its cross-sectional design. Even though sample size was large enough to explore the link between inflammation, cART-use, $mtDNA_{pl}$ and platelet function, it lacked power to confirm a possible link between $mtDNA_{pl}$ levels and clinical outcomes such as non-AIDS co-morbidities and NRTI-related adverse events. Although we observed reduced oxygen consumption in individuals with reduced $mtDNA_{pl}$, the overall reduction of $mtDNA_{pl}$ in PLHIV prevented to investigate functional consequences of $mtDNA_{pl}$ depletion in PLHIV. Furthermore, we did not perform immunofluorescence confocal or transmission electron microscopy experiments in the current study. In addition, age and sex differed substantially between cohorts, with an effect of age on mtDNA content in the uninfected cohort. We explored multiple methods to account for these differences using adjusted models and subgroup analyses. The subgroup of above >40 yrs revealed a significant difference between PLHIV and controls supporting the independent correlation of HIV infection in the age-sex adjusted model. Finally, our study included mostly Caucasian men limiting generalization of the findings to women and non-Caucasians.

In conclusion, PLHIV under long-term cART have reduced platelet mtDNA content and abnormalities in platelet mitochondrial respiration, which may possibly contribute to platelet dysfunction. Given the key role of platelets and mitochondria in the pathophysiology of long-term complications of HIV, interventions targeting platelet mitochondria, such as introducing NRTI sparing regimens, should be considered.

Methods

Patient selection

This cross-sectional, single center, prospective study was performed at the Radboud university medical center, a tertiary teaching hospital in The Netherlands. This study is part of the Human Functional Genomics Project (HFGP; www.humanfunctionalgenomicsproject.org) and was conducted in accordance with the Declaration of Helsinki after approval of the ethics committee (CMO Arnhem-Nijmegen, The Netherlands; NL42561.091.12, 2012/550). No animal experiments were performed in the current study. Adult HIV-1-infected individuals receiving cART for at least six months were included after providing written informed consent. Other inclusion criteria were a suppressed viral load (<200 copies/mL). Exclusion criteria included, use of P_2Y_{12} receptor antagonists (platelet inhibitor), an active hepatitis B or C infection and/or signs of other active intercurrent infection other than HIV-1 (e.g. fever in last week or antibiotic-use in last 4 weeks). Healthy controls were concurrently included throughout the duration of inclusion of PLHIV. Exclusion criteria were use of medication (excluding oral contraceptives or paracetamol) and/or signs of an active infection in the last month. Clinical data was collected by extracting data from electronic medical record (Epic

Systems, Verona, WI, USA). History of cART use was extracted from the Dutch HIV registry (Stichting HIV-monitoring). In a separate validation experiment for mitochondrial function, five virally suppressed male PLHIV (45-60yrs) who were not using statins or acetylsalicylic acid (ASA), were enrolled together with five age and sex matched controls.

Platelet count and function

Platelet count and parameters were determined using an automated hematology analyzer (Sysmex, Kobe, Japan). Platelet reactivity was determined in citrated whole blood (3.2% sodium citrate, Becton Dickinson, Franklin Lakes, NJ, USA) using a flow cytometry based assay as previously described between 1-3 hours after blood collection [52]. Platelets were ex vivo stimulated with ADP (1.2 and 125uM; Sigma-Aldrich, Zwijndrecht, The Netherlands) and CRP-XL (a kind gift from Prof. Farndale, Cambridge, UK) for 20 min at room temperature. Platelets were stained using anti-CD61 (Beckman Coulter, Brea. CA, USA), anti-P-selectin (Biolegend, San Diego, CA, USA) and anti-fibrinogen (DAKO, Santa Clara, CA) antibodies and fixated in 0.2% paraformaldehyde. Platelets were identified based on Size (FSC), granularity (SSC) and their expression of CD61. Degranulation was determined as the membrane expression of α-granule protein P-selectin and platelet aggregation was quantified as the amount of fibrinogen binding to the activated integrin αIIbβ3. Platelet reactivity was measured on a FC500 flow cytometer (Beckman Coulter, Brea, USA). Data were extracted using Kaluza 2.1 (Beckman Coulter), normalized against quality controls to ensure measurement stability and are expressed as median fluorescence intensity (MFI). A gating strategy is provided in Supplemental Figure 1.

Platelet isolation

Platelet rich plasma (PRP) was obtained from citrated plasma (Vacutainer, Beckton-Dickinson) after centrifugation 156g for 15min without brake at room temperature (RT). Samples were processed within 2 hours of blood collection. Platelet count in PRP was measured using an automated hematology analyser (Sysmex, Kobe, Japan). Washed platelets were obtained as previously described. [53] In short, PRP was supplemented with acid citrate dextrose (10%) and prostaglandin I_2 and washed twice with Hepes tyrode's buffer using centrifugation (330g, 20min).

Platelet microparticles

Peripheral blood was centrifuged at 1000g for 5 min. Plasma was then centrifuged at 1500g for 20 min to obtain platelet poor plasma (PPP). 1ml of PPP was centrifuged for 30 min at 20.000g to pellet microparticles (MPs). The MP pellet washed once in 500 µL calcium-free HBS complemented with 0.2% bovine serum albumin (pH 7.3) by 45 min centrifugation at 20.000g. MPs were labeled with MitoTracker Deep Red (200 nM; Invitrogen, Breda, The Netherlands) in HBS at room temperature containing calcium and subsequently stained with anti-CD61, anti-CD62p, Annexin-V (all Biolegend), anti-CD45 and anti-CD41 (both Beckman Coulter). MPs were analyzed using a Cytoflex flow cytometer (Beckman Coulter) including the sensitive violet Side Scatter (405nM; VSSC) and FSC for detection of ultrasmall particles (1µm).[54] Platelet MPs (PMPs) were selected based on aforementioned markers. Counting beads (Sphero Nano fluorescent, Spherotech, Fulda, Germany) of different sizes were included for reference to correct for concentration and size. All solutions for PMP isolation and staining were centrifuged at 20.000g for 20 min to remove (fluorescent) aggregates. A gating strategy is provided in Supplemental Figure 2.

mtDNA quantification and mitochondrial function

After platelet count measurement of every PRP sample, 500µL PRP was pelleted by centrifugation (2000g, 10min at RT without brake) and lysed using Triton –X100 0.1% for mtDNA quantification. $MtDNA_{pl}$ was measured by real time Quantitative PCR using Mitotox Quickscan (Primagen, Amsterdam, Netherlands), Advanced SYBR green Super mix (Bio-rad, Hercules, CA, USA) and CFX96 Real time Detection System (Bio-Rad) according to manufacturer's instructions. The kit includes primers and calibrators for quantification. A calibration curve with known mtDNA concentration was concurrently measured on every plate to ensure stability and quantification. A dilution curve with PRP showed a high correlation coefficient for mtDNA and platelet concentration. mtDNA copies per platelet ($mtDNA_{pl}$) are calculated by dividing mtDNA copy number per well by platelet count in 500µl of PRP. Contamination of leukocyte and erythrocyte was low <1:10.000. Samples with low platelet counts in PRP (below 50.10^12/mL) and erythrocyte/leukocyte contamination were excluded (n=2). Mitochondrial respiration was quantified using the 96-well format Seahorse extracellular flux analyzer (Agilent, MA, USA). Washed platelets were plated to 60.000 platelets/µL and seeded onto Cell-Tak coated XF96 microplates (Corning, Corning, NY, USA). A mitochondrial stress test was performed as described eslewhere[42] using oligomycin (1µM, ATP synthase inhibitor (complex V), reducing mitochondrial respiration), Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP;1µM, uncoupling agent causing maximum mitochondrial respiration), antimycin A (2.5 µM, Complex III inhibitor inhibiting mitochondrial respiration) and 2-deoxy-D-glucose (2DG; 40mM, inhibits glycolysis) after ex vivo stimulation (all Sigma). Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were determined before and after injection of platelet agonists. Samples were measured in five replicates with three measurements after every injection. For mitochondrial respiration measurements using Seahorse extracellular flux analyzer, significant outliers were excluded from analysis if measurement was 2x SD below the mean value in every timepoint with a maximum of one of the five replicates.

Mitochondrial membrane potential was determined using Tetramethylrhodamine ethyl ester (TMRE, Sigma; 100nM at 37 °C for 20min). A negative control was generated for every sample using FCCP (1µM at 37 °C for 10min). Mitochondrial reactive oxygen species (ROS_m) were detected using the cationic probe MitoSOX Red (Invitrogen, Carlsbad, CA, USA (2.5µM at 37 °C for 30min). Both probes were quantified in CD61+ cells using a Cytoflex flow cytometer (Beckman Coulter).

Plasma markers

Concentrations of three plasma markers of platelet activation: chemokine (C-X-C motif) ligand 4 (CXCL4 also known as platelet factor 4), CXCL7 (also known as beta-thromboglobulin) and chemokine (C-C motif) ligand (CCL5; also known as RANTES) were determined by ELISA (R and D systems, Minneapolis, USA) according to the manufacturer's instructions in citrated PPP.[52] In addition, markers of persistent inflammation, high-sensitive C-reactive protein (hsCRP), sCD163 and sCD14, were measured using ELISA (Quantikine, R and D systems) according to the manufacturer's instructions in EDTA plasma.

Statistical analyses

Data were analyzed by independent T-test or Mann-Whitney U test. Pearson's correlations coefficient was used for univariate correlation analyses, unless otherwise stated. An inverse rank-based normalization was performed for non-normal data (eg. mtDNA in platelets). A multivariate linear regression model was used to correct for age, body mass index (BMI) and sex. Several sensitivity analyses using subgroups (males only and above 40 years) were performed to explore for possible confounding. Principal component analysis (PCA) was performed using singular value decomposition to summarize platelet function and correlate with $mtDNA_{pl}$. cART-use was calculated as days on a certain drug, cumulative use of multiple drugs of the same class were combined. R studio (CRAN project) and Graphpad Prism version 5.03 were used for analyses.

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Supplementary Table 1. Platelet hematology indices, platelet activation parameters and markers of inflammation in individuals age >40 years.

Platelet indices	200HIV (n=173)	50FG (n=22)	p -value
Platelet count (10^9/L)	2.6 [2.0, 3.1]	2.8 [2.4, 3.0]	0.483
MPV (fL)	10.1 [9.7, 10.5]	10.9 [10.3, 11.4]	0.007
IPF (%)	3.2 [2.4, 4.5]	5.9 [3.4, 9.4]	0.008
Baseline fibrinogen binding (MFI)	2.0 [1.6, 2.2]	2.2 [2.1, 2.3]	0.002
Baseline P-selectin expression (MFI)	2.6 [2.0, 3.2]	2.9 [2.8, 3.1]	0.028
CCL5 (ng/ml)	2.66 [1.63, 4.23]	2.07 [1.60, 3.19]	0.245
Platelet factor 4 (ng/ml)	452.5 [294.5, 715.2]	324.0 [258.3, 462.4]	0.164
CXCL7 (ng/ml)	288.3 [180.9, 455.1]	213.9 [189.1, 260.3]	0.173
sCD14 (ng/ml)	2207.4 [1787.8, 2677.2]	1952.2 [1741.9, 2134.7]	0.062
sCD163 (ng/ml)	741.6 [547.3, 905.6]	516.8 [442.7, 684.2]	0.01
hsCRP (ng/ml)	1493.4 [608.4, 3051.2]	812.6 [382.2, 1671.0]	0.073

MPV: mean platelet volume. IPF: immature platelet fraction as a percentage of platelet count. (Sysmex, Kobe, Japan). Baseline platelet aggregation measured as Fibrinogen binding by flowcytometry in median fluorescence intensity (MFI). Baseline platelet degranulation measured as P-selectin expression by flow cytometry by MFI. sCD14: Serum levels of CD14, a marker of monocyte activation. sCD163: Serum levels of CD163, a monocyte- and macrophage-specific scavenger receptor, hsCRP: high sensitive C-reactive protein. Data depicted as median [interquartile range] and analyzed using Mann–Whitney

Supplementary Table 2. Platelet hematology indices, platelet activation parameters and markers of inflammation in males only.

Platelet indices	200HIV (n=191)	50FG (n=34)	p -value
Platelet count (10^9/L)	260 [200, 310]	260 [220, 310]	0.483
MPV (fL)	10.1 [9.7, 10.5]	10.9 [10.3, 11.4]	0.007
IPF (%)	3.3 [2.6, 4.7]	5.0 [3.2, 7.0]	0.007
Baseline fibrinogen binding (MFI)	2.0 [1.6, 2.2]	2.2 [2.0, 2.4]	<0.001
Baseline P-selectin expression (MFI)	2.7 [2.1, 3.2]	3.0 [2.8, 3.2]	0.008
CCL5 (ng/ml)	2.66 [1.63, 4.23]	2.07 [1.60, 3.19]	0.503
Platelet factor 4 (ng/ml)	458.4 [295.3, 726.1]	494.0 [333.1, 858.2]	0.39
CXCL7 (ng/ml)	290.4 [187.0, 452.8]	317.1 [214.4, 542.5]	0.466
sCD14 (ng/ml)	2155.2 [1780.7, 2672.7]	1851.0 [1614.1, 2172.7]	0.006
sCD163 (ng/ml)	700.2 [523.9, 900.0]	532.1 [418.3, 620.5]	0.002
hsCRP (ng/ml)	1451.7 [608.4, 2702.7]	675.9 [166.5, 1309.3]	0.003

MPV: mean platelet volume. IPF: immature platelet fraction as a percentage of platelet count. (Sysmex, Kobe, Japan). Baseline platelet aggregation measured as Fibrinogen binding by flowcytometry in median fluorescence intensity (MFI). Baseline platelet degranulation measured as P-selectin expression by flow cytometry by MFI. sCD14: Serum levels of CD14, a marker of monocyte activation. sCD163: Serum levels of CD163, a monocyte- and macrophage-specific scavenger receptor, hsCRP: high sensitive C-reactive protein. Data depicted as median [interquartile range] and analyzed using Mann–Whitney.

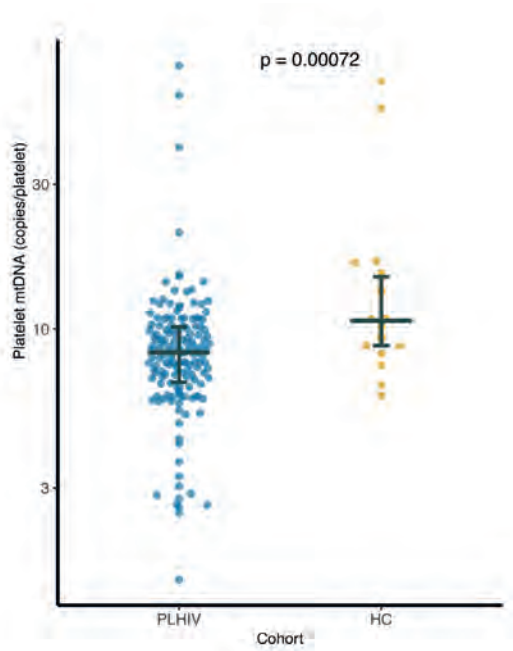
Supplementary Table 3. Pearson's correlation coefficient between platelet mtDNA and clinical parameters.

	Platelet mtDNA content	
	R	p-value
CD4 NADIR	0.005	0.95
CD4 COUNT	-0.02	0.76
CD4/CD8 ratio	0.03	0.68
cART duration	-0.03	0.63
HIV infection duration	-0.086	0.23

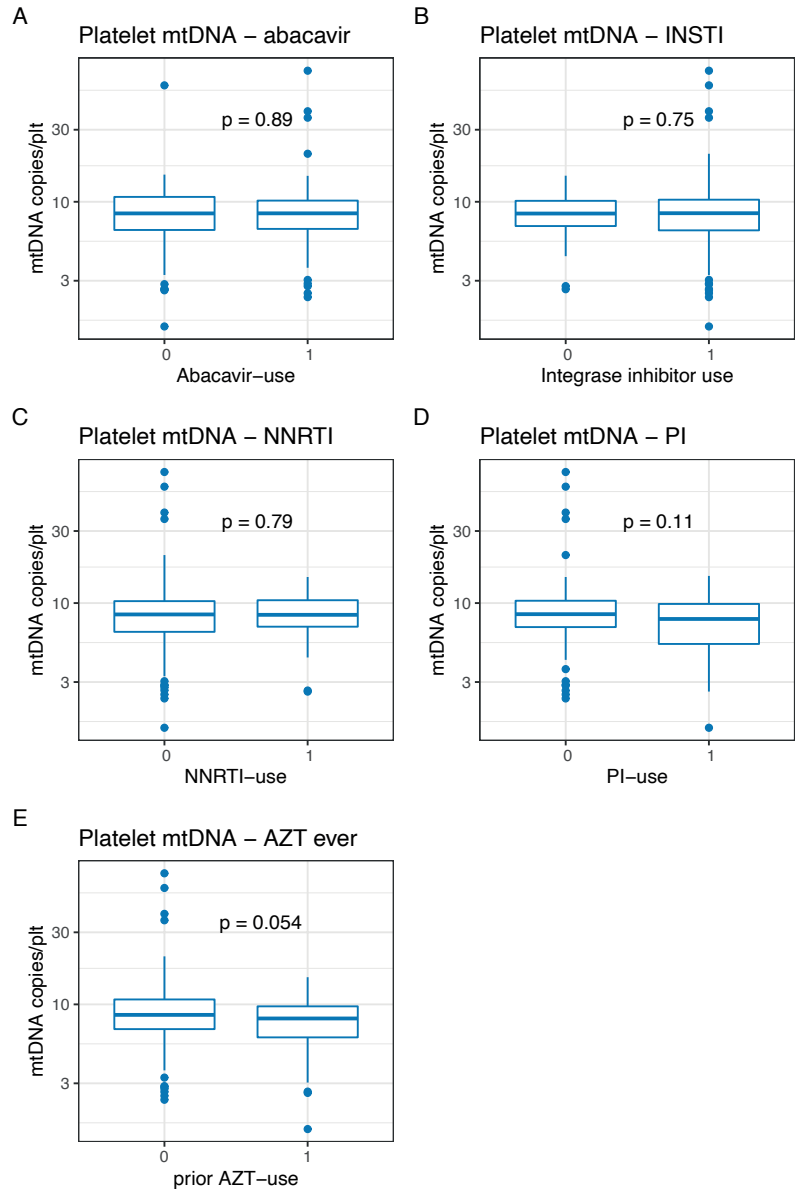
mtDNA was normalized using inverse rank-based transformation.

Supplementary Table 4. Pearson's correlations between Principal component 1 and Principal component 2 with clinical variables and inflammation.

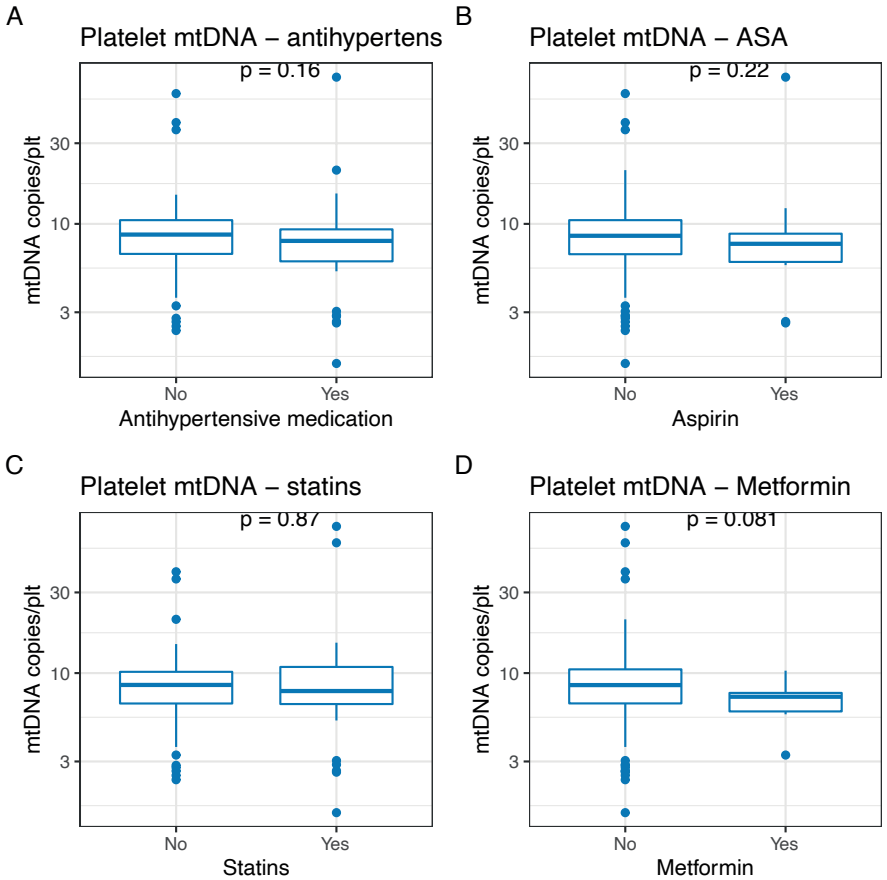
	Principal component 1		Principal component 2	
	R	p-value	R	p-value
CD4 NADIR	0.13	0.095	0.09	0.24
CD4 COUNT	0.05	0.56	0.07	0.34
CD4/CD8 ratio	0.09	0.26	-0.02	0.79
cART duration	-0.09	0.24	0	0.96
HIV infection duration	-0.04	0.56	0.02	0.83
sCD14	-0.05	0.43	0.02	0.81
sCD163	-0.1	0.15	-0.09	0.2
hsCRP	-0.04	0.53	0.01	0.88



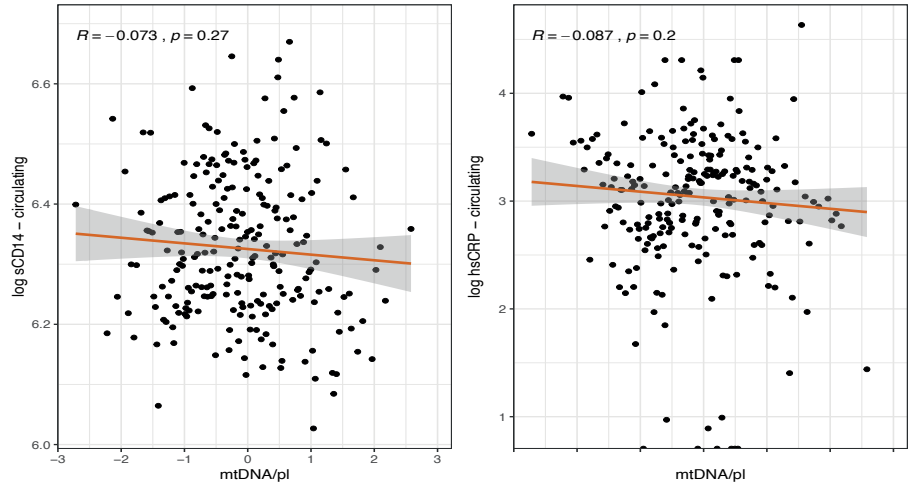
Supplementary figure 1. Platelet mtDNA levels as copies/platelet. Subgroup analysis of individuals Age >40 years (people living with HIV (PLHIV): n=173, healthy controls (HC): n=22). Data are shown as dotplot with error bars median and interquartile range (IQR). Data were analyzed using unpaired Student's T-test. People living with HIV (PLHIV); healthy controls (HC).



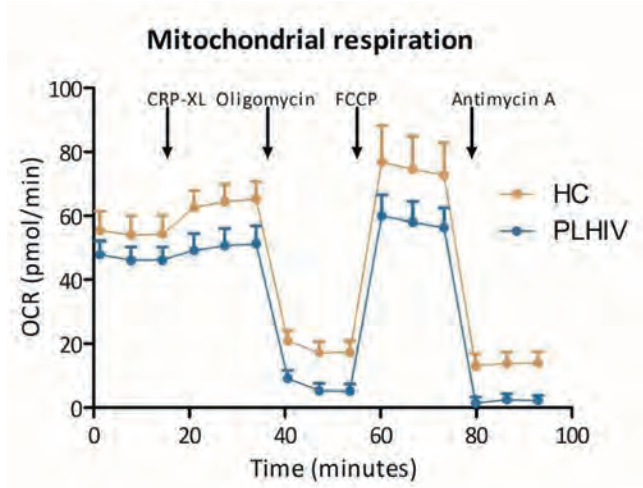
Supplementary figure 2. Platelet mtDNA levels as copies/platelet. (A) abacavir-use (B) Integrase inhibitor use (INSTI) (C) non-nucleoside reverse transcriptase inhibitor use (NNRTI) (D) Protease inhibitor use (PI). (E) History of Zidovudine (AZT) use. Data were analyzed using Mann–Whitney.



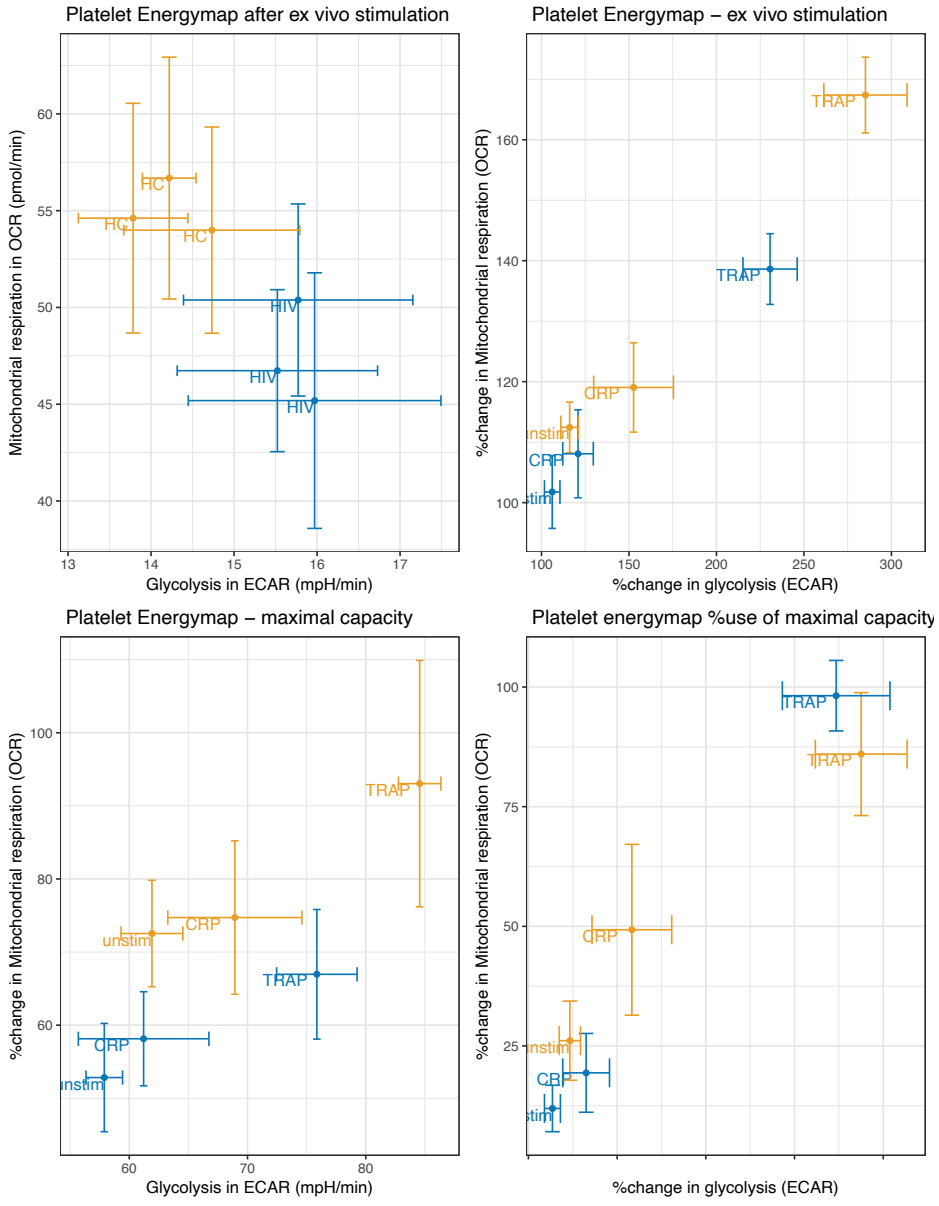
Supplementary figure 3. Platelet mtDNA levels stratified by use of co-medication. (A) antihypertensive drugs (eg. ACE-inhibitor, diuretics) (B) low-dose Acetylsalicylic acid (80mg/day; ASA; Aspirin) (C) Cholesterol lowering drug class statins (D) Anti-diabetics drug Metformin. Data were analyzed using Mann–Whitney.



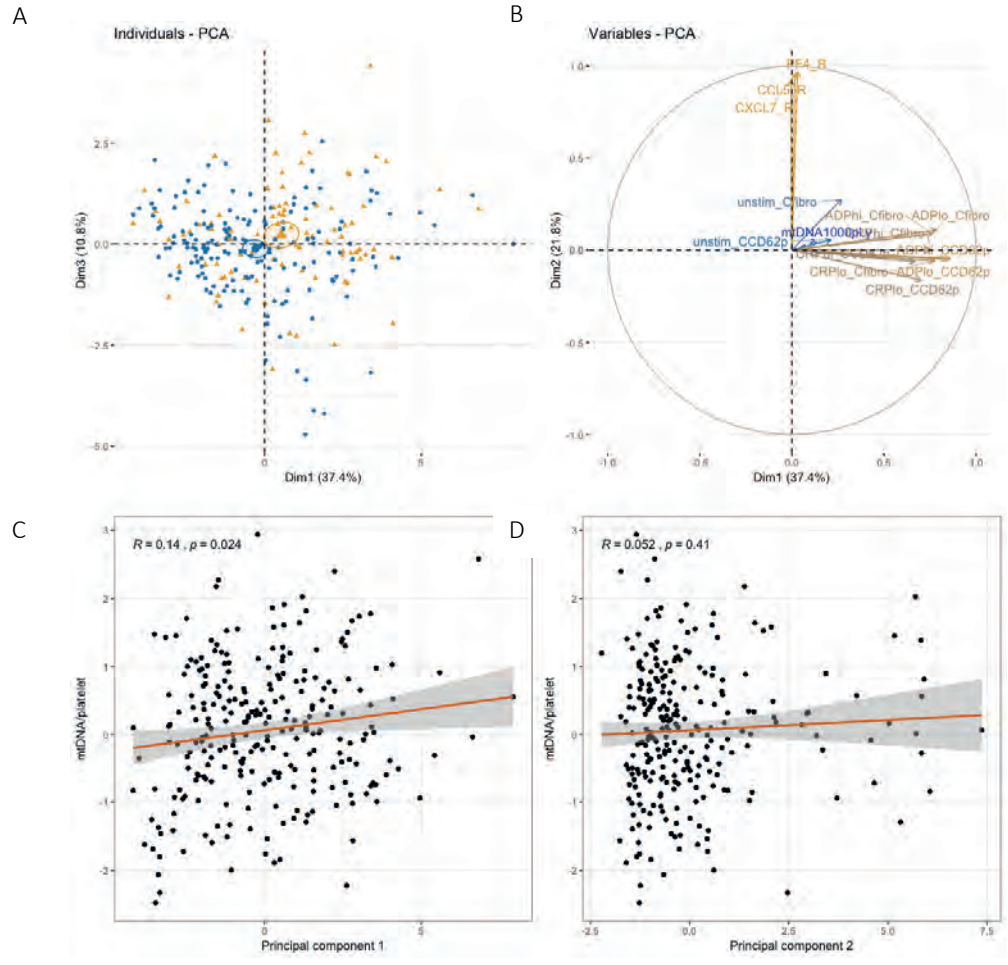
Supplementary figure 4. Correlation between inflammation and platelet mtDNA. (A) soluble CD14, a marker of in vivo monocyte activation (B) high-sensitive C-reactive Protein (hsCRP). sCD14 and hsCRP were log-transformed. mtDNA/pl were transformed using an inverse rank-based normalization. Data were analyzed using Pearson's coefficient.



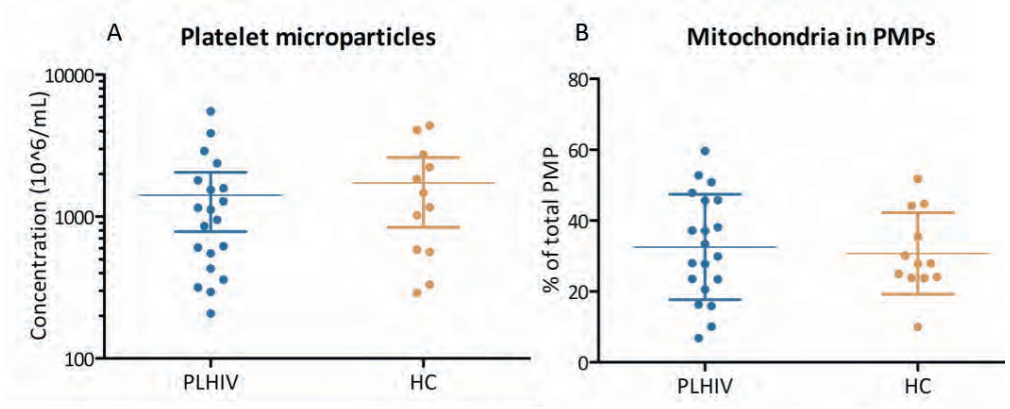
Supplementary figure 5. Platelet energy phenotype determined by the Seahorse extracellular flux analyzer after CRP-XL stimulation. Samples were measured with five replicates, 1.10^7 washed platelets per well. Five age-and sex-matched donors per group. Real-time mitochondrial respiration depicted as oxygen consumption rate (OCR) at 18 timepoints. Four agonists or inhibitors were added in the following order: 1) collagen-related peptide (CRP-XL; 50ng/mL or medium causing platelet activation 2) Oligomycin which inhibits cellular ATP production 3) Carbonyl cyanide-4-(trifluoromethoxy) phenylhydrazone (FCCP), an uncoupling agent causing maximum oxygen consumption through complex IV and 4) Antimycin A which inhibits all mitochondrial respiration (C) real-time glycolysis as extracellular acidification rate (ECAR).



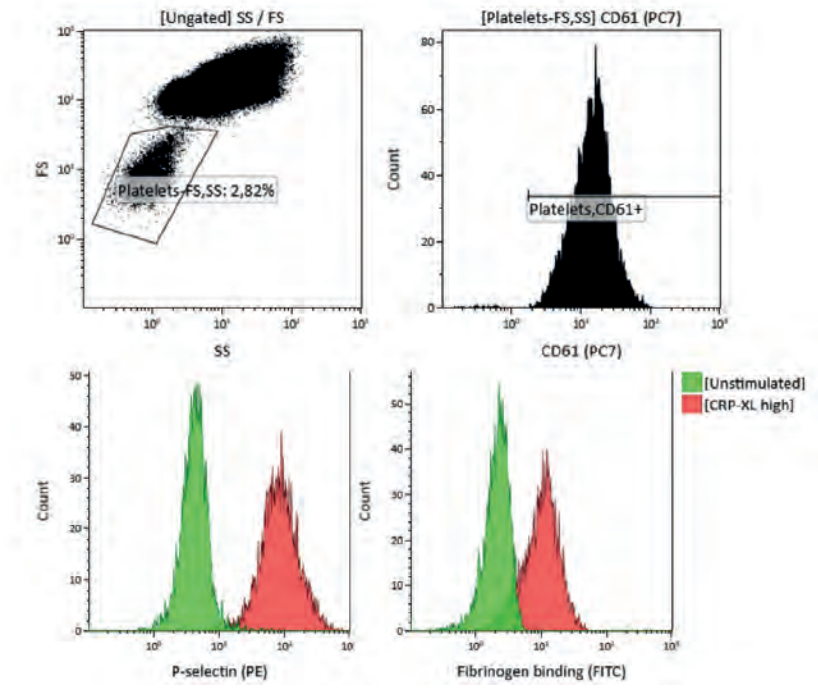
Supplementary figure 6. Platelet energy phenotype after ex vivo stimulation. Platelet energy phenotype was determined using a Seahorse XFe96 Analyzer. (A) baseline Oxygen consumption rate (OCR) in pmol/min and extracellular acidification rate (ECAR) in mpH/min at baseline. (B) Change in OCR and ECAR after ex vivo stimulation with Trombin (TRAP; 50uM), Collagen-related peptide (CRP-XL; 50ng/mL) or unstimulated. (C) Maximal mitochondrial respiration capacity after FCCP (1µM) stimulation and maximal glycolysis capacity after oligomycin (1µM). (D) Percentage used after ex vivo stimulation of total energy capacity. Data shown are means with ± SEM.



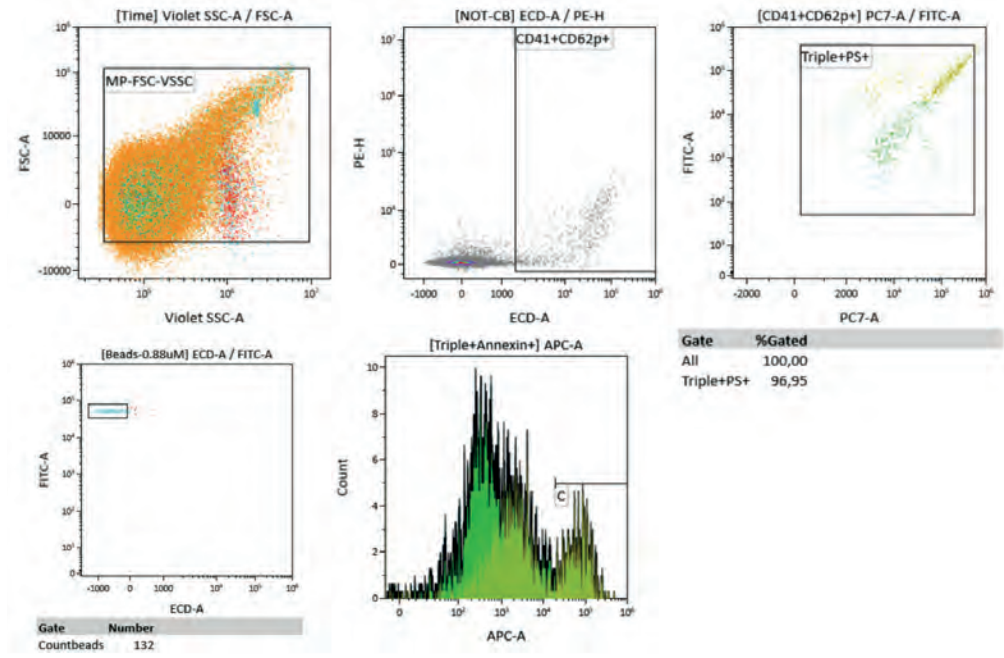
Supplementary figure 7. Principal component analysis (PCA) of platelet activation and reactivity markers. (A) Coordinates of individuals on PC1 (x-axis) and PC2 (y-axis). All platelet activation markers (plasma levels of CCL5, CXCL7 and PF4; unstimulated P-selectin expression and unstimulated fibrinogen binding) and platelet reactivity markers (P-selectin expression and fibrinogen binding after ex vivo stimulation) were used in this PCA. (B) Figure shows the direction of the used variables. Adenosine diphosphate (ADP), Fibrinogen binding (Fibro), P-selectin (CD62p). (C-D) correlation between PC1/PC2 and mtDNA/pl. Platelet mtDNA (Y-axis) was normalized using inverse rank-based transformation.



Supplementary figure 8. Platelet microparticles and platelet mitochondrial release. (A) Platelet microparticle (PMP) count were determined by CD61, CD41, P-selectin (CD62p) and Annexin V expression and normalized to beads count. (B) The percentage of mitochondria positive PMPs were measured using Mitotracker Green. 17 HIV infected individuals and matched controls were used for this experiment. No significant differences could be observed by using the student's T-test. Data are depicted as mean ±standard deviation.



Supplementary figure 9. Gating strategy using Kaluza 2.1 (Beckman Coulter) for platelet reactivity. First platelets were gated based on size (Forward scatter), granularity (Sideward scatter) and CD61 positivity. Thereafter P-selectin expression and Fibrinogen binding were determined upon stimulation.



Supplementary figure 10. Platelet microparticle (MP) gating strategy. MPs were labelled with MitoTracker Deep Red (200 nM; Invitrogen, Breda, The Netherlands) in HBS at room temperature containing calcium and subsequently stained with anti-CD41 (Beckman Coulter), anti-CD61 (Biolegend, San Diego, CA), anti-CD62p (Biolegend), Annexin-V (Biolegend) and anti-CD45 (Beckman Coulter). MPs were analyzed using an Cytoflex flow cytometer (Beckman Coulter, Brea, CA) including the sensitive violet Side Scatter (405nM; VSSC) and FSC for detection of ultrasmall particles (1µm). Platelet MPs (PMPs) were selected based on aforementioned markers.

Chapter 7

A Switch to a raltegravir containing regimen does not lower platelet reactivity in HIV-infected individuals; a randomized controlled trial

Wouter van der Heijden, Reinout van Crevel, Philip de Groot, Rolf Urbanus, Hans Koenen, Marjolein Bosch, Monique Keuter, Andre van der Ven, Quirijn de Mast

AIDS; 2018 Nov 13;32(17):2469-2475

Abstract

Platelet hyperreactivity and increased platelet-monocyte aggregation (PMA) are associated with increased cardiovascular risk and inflammation. In a previous cross-sectional study, individuals using a raltegravir (RAL)-based regimen were found to have reduced platelet reactivity and PMA compared with other antiretroviral regimens. Our aim was to investigate whether switching from a non-integrase inhibitor regimen to a RAL-based regimen reduces platelet reactivity or PMA. An investigator initiated, single-centre, prospective randomized, open-label, blinded endpoint trial. Forty HIV-infected adults using a non-integrase inhibitor containing regimen with undetectable viral load were randomized to either continue their regimen or switch to a RAL-based regimen for 10 weeks, continuing the same backbone. The primary outcome was the change in platelet reactivity at week 10, which was determined as the expression of the platelet activation marker P-selectin and binding of fibrinogen before and after ex-vivo stimulation with different platelet agonists. Secondary outcomes included PMA, plasma markers of platelet activation and markers of inflammation and immune cell activation. Twenty-one participants were enrolled in the continuation group and 19 in the RAL group. Baseline characteristics were comparable between groups. There were no differences in the change in platelet reactivity to either platelet agonist at week 10, nor in plasma markers of platelet activation. PMA, C-reactive protein, T-cell activation (CD38+HLA-DR+) and monocyte (CD14+CD16+) subsets. Switching a non-integrase inhibitor containing regimen to a RAL-based regimen does not reduce platelet reactivity, platelet-leukocyte aggregation, inflammation and immune activation in virologically suppressed HIV-infected individuals.

Introduction

Cardiovascular disease is a leading cause of morbidity and mortality in HIV-infected individuals[1]. Persistent inflammation is thought to be a major factor in this excess risk[2]. Mounting evidence supports that platelets are important regulators of inflammation and are involved in both onset and progression of atherosclerosis, next to acute cardiovascular events[3,4]. Activated platelets release a wide range of inflammatory mediators and form aggregates with leukocytes, most notably via platelet P-selectin[4,5]. Platelet-monocyte aggregation (PMA) is a sensitive marker of *in vivo* platelet activation[6] and renders monocytes more pro-inflammatory[7,8].

Different studies have shown that both antiretroviral naïve and treated HIV-infected individuals have more activated and reactive platelets and increased PMA compared to HIV-uninfected individuals[9-13], although other studies yielded opposite results[14,15]. In addition, HIV-infected patients with an acute coronary syndrome had higher platelet reactivity and residual platelet hyper-reactivity on dual anti-platelet therapy compared with HIV-uninfected patients[16]. Together, these data suggest that platelets are a potential target to reduce persistent inflammation and the excess CVD risk in HIV.

Our group recently found in a cross-sectional study that individuals on a raltegravir (RAL)-based regimen had reduced platelet reactivity and PMA compared to those on a non-integrase inhibitor-based regimen[12]. Therefore we performed a randomized controlled trial to study whether a switch to a RAL-based regimen leads to a reduction in platelet reactivity, platelet-leukocyte aggregation, inflammation and immune activation.

Methods

This investigator initiated, single centre, prospective randomized open-label, blinded end-point trial (NCT02383355) was performed at the Radboud university medical centre, a tertiary teaching hospital in the Netherlands. This study was conducted in accordance with the Declaration of Helsinki after approval of the ethics committee (CMO Arnhem-Nijmegen, The Netherlands; NL5068109114). Adult HIV-1-infected individuals receiving a non-integrase inhibitor (INSTI) containing regimen with a standard backbone of two NRTIs for at least six months were included after providing written informed consent. Other inclusion criteria were a suppressed viral load (<50copies/mL) for at least 6 months and a CD4 cell count >300cells/mL. Exclusion criteria included the use of platelet inhibitors, signs of an active infection other than HIV-1, estimated glomerular filtration rate below 50 ml/min, liver enzyme abnormalities, known genotypic resistance to any current ART component, history

of virological failure (RNA blips <500copies/mL with subsequent suppression were allowed) and pregnancy or breastfeeding. Randomisation was performed using a computer generated block randomization algorithm (CASTOREDC, The Netherlands).

Participants were randomised 1:1 to either switch to RAL 400mg twice daily continuing their current backbone therapy (RAL-group) or continuing their current regimen (CONT-group). Participants were followed for ten weeks with study visits at week 4 and week 10. At study visits, blood was obtained for platelet and inflammation markers and adherence and adverse events were recorded. The primary outcome was the change in platelet reactivity between week 0 and 10. This was defined as the expression of the platelet activation marker P-selectin, a marker for platelet degranulation, and the binding of fibrinogen to the activated fibrinogen receptor (integrin $\alpha_{IIb}\beta_3$), a marker for platelet aggregation, following *ex vivo* stimulation. Secondary outcomes included changes in platelet-leukocyte aggregation, monocyte subsets, T-cell activation and soluble markers of inflammation, platelet activation and plasmatic coagulation.

Platelet reactivity was determined in citrated whole blood using a flow-cytometry based assay as previously described[12] (supplementary figure 1). Platelet-leukocyte aggregation, monocyte subsets and T-cell activation were measured at baseline and after ten weeks in whole blood on a NAVIOS flow-cytometry as previously described[17] (supplementary figure 2). Plasma concentrations of platelet factor 4 (PF4), beta-thromboglobulin (beta-tg/CXCL7), high-sensitive C-reactive protein (hsCRP) and thrombin antithrombin complexes were measured using ELISA as previously described[18]. All laboratory procedures are described in detail in the supplementary information.

The primary outcome was analyzed by calculating the change in platelet reactivity from baseline to end-of-study time point (week 10) and comparing the groups using independent Student's T-test. Secondary outcomes were analyzed by independent T-test or Mann-Whitney test depending distribution. Intention-to-treat analysis was performed and data are expressed as mean with standard deviation (SD) or median with interquartile range (IQR) depending distribution. Sample size estimation was based on the data of previous cross-sectional study[12]. We anticipated ADP-induced P-selectin expression in the RAL-group to decrease from a MFI of 19 to 14 (SD 5.2). Demonstrating this difference with a power of 80% and α=0,05 required 18 individuals per group. Consequently, we have included 20 per group. SPSS Statistics 22 and Graphpad Prism 5 were used for analyses.

Results

Patient recruitment started in March 2015 and the last patient completed the study in March 2016. A flow diagram of the study is presented in supplementary figure 3. Nineteen participants were randomized to switch to RAL without changing their NRTI backbone therapy (RAL-group) and 21 continued their current regimen (CONT-group). One patient started aspirin after informed consent, but before treatment allocation, and was therefore excluded. Thirty-eight subjects completed the total follow-up period of 10 weeks. Two subjects discontinued RAL at week 4 due to grade I adverse events (mild sleeplessness and headache) and this time point was included as the end-of-study (EoS) time point. Overall, treatment was well tolerated and no grade III-IV adverse events were recorded.

Baseline characteristics, which are presented in supplementary table 1, were similar between the groups. Overall, 97.5% of participants were male with a median age of 48yrs (IQR:43-54) in the RAL-group and 49yrs (IQR:42-61) in the CONT-group. The ART backbone consisted of TDF/FTC (RAL:13 (68,8%), CONT:17 (81%)) or ABC/3TC (RAL:5 (26.3%), CONT:3 (14.3%)). Seventy-nine percent of participants in the RAL-group were using a NNRTI-based regimen at enrolment. All subjects had an undetectable viral load (VL;<50 copies/mL) at baseline. Two subjects in the RAL-group had a detectable VL during follow-up: one subject had a VL of 90 copies/mL at week 4 with undetectable VL at week 10; the second subject had a VL of 2000 copies/mL at week 10 due to suboptimal adherence.

Platelet reactivity at baseline, as measured by P-selectin expression and fibrinogen binding after *ex vivo* stimulation with ADP, CRP-XL and TRAP-6 was similar between groups (Figure 1a-b). Additionally, there were no differences observed in platelet reactivity at baseline between subjects on abacavir or tenofovir as backbone therapy (supplementary table 2). At week 10, there was no difference between both groups in the primary outcome parameters, *i.e.* the change in platelet reactivity parameters (figure 1c-d).

This was supported by results from the plasma markers. Plasma levels of the platelet activation markers beta-thromboglobulin/CXCL7 and PF4 were similar at baseline and did not change in either group at week 10 (figure 2a-b). Concentrations of the plasmatic coagulation marker thrombin antithrombin complexes and the inflammation marker hsCRP also remained unchanged at week 10 compared to baseline (figure 2a-b).

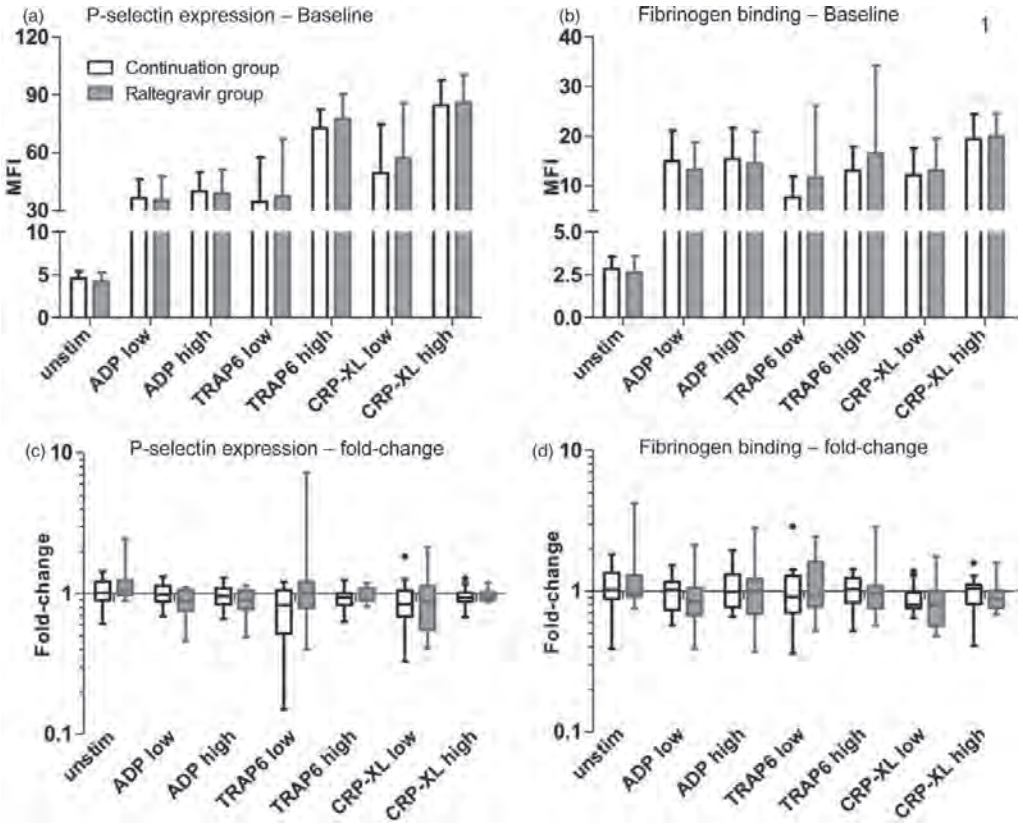


Figure 1. Platelet reactivity at baseline and end of study. Platelet reactivity was determined by measuring platelet P-selectin expression and fibrinogen binding in unstimulated samples and after ex vivo stimulation with three agonists at two concentrations. **A-B**) P-selectin expression and fibrinogen binding at baseline. Data depicted as mean and standard deviation (sd). **C-D**) Fold-change in P-selectin expression and fibrinogen binding from baseline to end of study. Data depicted according to Tukey (median, interquartile range, range excluding outliers defined as 3/2 times outer quartile and outliers are depicted as dots). No significant differences were found between both groups using independent Student's T-test. Adenosine diphosphate (ADP; low: 7.8 µM and high 125 µM), Thrombin Receptor Activator for Peptide 6 (TRAP6; low: 9.8 µM and high: 156 µM) or cross-linked collagen-related-peptide (CRP-XL; low: 27.33ng/L or high: 655.7ng/L).

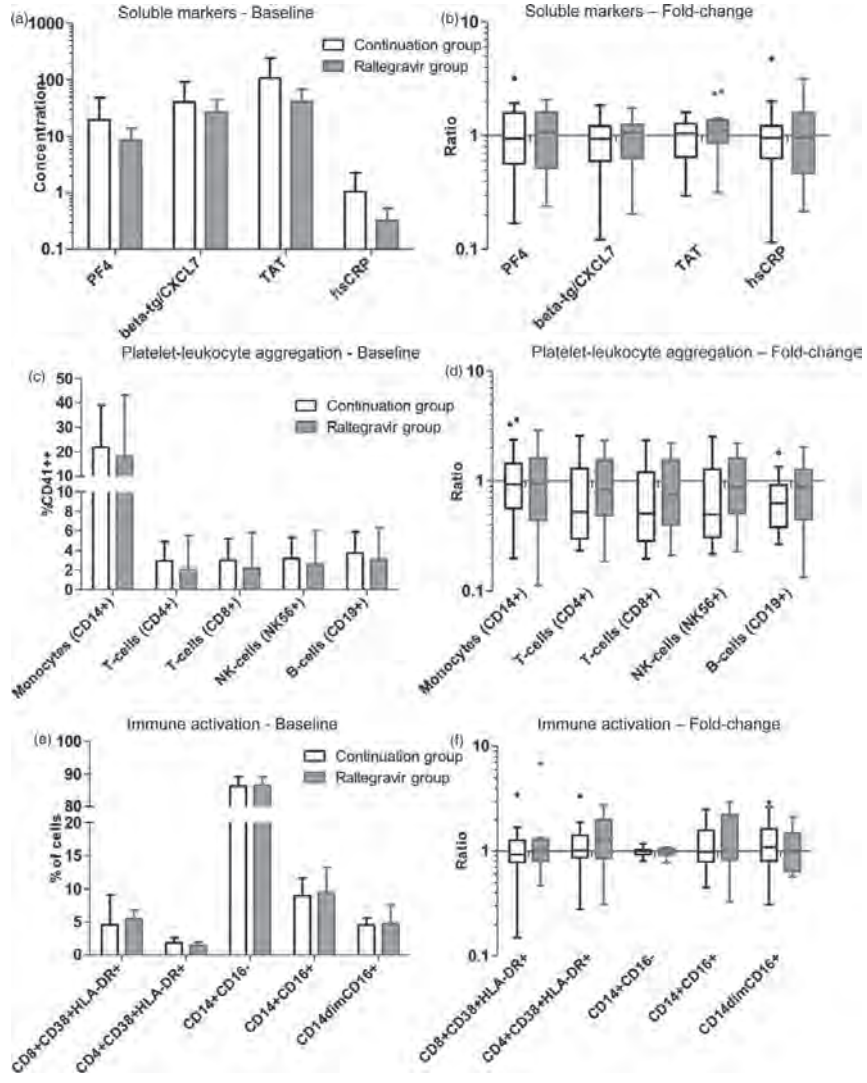


Figure 2. Soluble markers and cellular markers of platelet activation, inflammation and immune activation. **A**) Plasma concentrations of platelet activation markers (platelet factor 4 (PF4, ng/mL) and beta-thromboglobulin (beta-tg/CXCL7, ng/mL)), plasmatic coagulation thrombin-antithrombin complexes (TAT, pM)) and high sensitive C-reactive protein (hsCRP, ug/mL)). Data are depicted as median with interquartile range (IQR). **B**) Fold-change from baseline to end of study. Data depicted according to Tukey (median, IQR, range excluding outliers defined as 3/2 times outer quartile and outliers are depicted as dots). **C**) Platelet-leukocyte aggregates were measured by flow-cytometry and are quantified by percentage (%) CD41-positive events on $CD14^+$ cells (monocytes), $CD4^+$cells, $CD8^+$cells, $CD56^+$cells (NK-cells) and B-cells ($CD19^+$cells). Data are shown per group as median with IQR. **D**) Fold-change in platelet-leukocyte aggregates from baseline to week 10. Data are depicted according to Tukey. **E**) Baseline values of T-cell activation were defined as the % of double positive ($CD38^+HLA\text{-}DR^+$) CD4 or CD8-cells of the total CD4+ or CD8+ population. Monocyte subsets are depicted as the % of the total $CD14^+$ cells. Classical monocytes ($CD14^+CD16^-$), intermediate monocytes ($CD14^+CD16^+$) and non-classical monocytes ($CD14^{dim}CD16^+$). Data are depicted as median with IQR. F) Fold-change in T-cell activation and monocyte subsets from week 0 to week 10. Data are depicted according to Tukey. No statistical differences were found using Mann-Whitney-U test.

Platelet-leukocyte aggregation in our study was most prominent for platelet-monocyte aggregation (RAL:18.2% $CD41^+$ of $CD14^+$cells (IQR:9.2-43.26), CONT:18.6% (IQR:11.6-23.9)) compared to platelets aggregating with lymphocyte subsets (RAL:2.5% (IQR:1.5-5.8), CONT: 2.8% (IQR:1.4-4.6)) (figure 2c). At baseline, no differences were observed between groups and platelet aggregation to monocyte and lymphocyte subsets did not differ significantly at week 10 (figure 2c-d). An increase in activated proinflammatory monocyte subsets ($CD14^+CD16^+$) is associated with cardiovascular disease[19,20]. Circulating classical monocytes ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and nonclassical monocytes ($CD14^{dim}CD16^+$) were similar at baseline and were not influenced by a switch to RAL in this study (figure 2e-f). T-cell activation measured by CD38 and HLA-DR co-expression was also not statistically different between the RAL-group and CONT-group at baseline or after 10 weeks of treatment (figure 2e-f).

Discussion

This randomized controlled trial demonstrates that switching from a non-INSTI containing regimen to a RAL-based regimen in virologically suppressed HIV-infected individuals does not reduce platelet reactivity, platelet-leukocyte aggregation and markers of immune activation, such as activation of monocytes and T-lymphocytes and concentrations of CRP and TAT. These findings are in accordance with a study by Martinez et al[21], which showed that switching from a boosted protease inhibitor (PI) to RAL for 48 weeks did not lead to a reduction of *in vivo* platelet activation measured by soluble P-selectin. Yet, it is in apparent contrast to previous findings from a cross-sectional study performed by our group showing that individuals on a RAL-based regimen had reduced platelet reactivity compared to those on a NNRTI or PI containing regimen[12]. This may be explained in different ways. First, the follow-up period of 10 weeks in the present study is relatively short, whereas the majority of patients in the cross-sectional study were on the same cART longer than 3 months. Although, platelet life-span is short (<10 days) and RAL intensification has shown effects on viral replication and plasmatic coagulation within 3 months[22,23], effects on megakaryocytes may take longer. Second, cross-sectional studies are prone for selection bias. The latter is especially relevant as available data on platelet reactivity in cART treated patients are contradictory, with some studies reporting increased platelet reactivity[9-13] while others reporting reduced reactivity[14,15].

Platelets are increasingly recognized as key effector cells in inflammation[4]. In turn, inflammation may also influence platelet reactivity[24]. Some studies have proposed that RAL may reduce inflammation more effectively than other treatment regimens[22,23]. Part of the inflammatory effects of platelets is mediated by their interaction with leukocytes[7,8]. Our data do not support the notion that switching to RAL reduces inflammation or immune activation. The effects of RAL on inflammation and immune activation in the literature are also controversial. In cART-intensification studies, addition of RAL reduced levels of the monocyte activation marker sCD14[25] and D-dimer in some studies[23], whereas no effect on innate immune activation or coagulation was observed in others[22,26,27]. In the study by Martinez et al[21], switching from a PI- to a RAL-based regimen decreased hsCRP and IL-6 levels, while D-dimer and soluble P-selectin levels remained similar. Conversely, a study in treatment-naive participants showed no consistent reduction of inflammation in RAL-treated patients compared to treatment with boosted PIs[28]. Abacavir has been shown to influence platelet function[29]. We did not find significant differences in platelet activation or reactivity between those on an abacavir or tenofovir-based backbone at enrolment. Also, the backbone was not changed during the trial, minimizing the possible confounding effects of abacavir on our outcome measures.

Our study has several limitations. Even though our study was correctly powered for the primary outcome (platelet reactivity), the sample size gave us limited statistical power to explore all secondary objectives in detail and to include sub-analyses exploring possible confounders. Secondly, the inclusion of only long-term, virologically suppressed individuals could have masked the possible beneficial effects of RAL, but mirrors the current chronically infected HIV population. Finally, our study included mostly men limiting generalization of the findings to women. The same applies to generalizability of these data to other INSTIs.

In conclusion, this study shows that switching to a RAL-based regimen does not reduce platelet reactivity, platelet-leukocyte aggregation, inflammation and immune activation in virologically suppressed HIV-infected individuals.

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Supplementary methods

Laboratory procedures

Platelet reactivity was determined in citrated whole blood (3.2% sodium citrate, Becton Dickinson) using a flow-cytometry based assay as previously described [1]. Whole blood was processed after a rest period of one hour and within three hours after collection to prevent iatrogenic platelet activation. In short, whole blood was added to saturating concentrations of anti-CD61 (Beckman Coulter, Brea, USA), anti-fibrinogen (DAKO, Belgium) and anti-CD62p (anti-P-selectin; BD Biosciences, San Jose, USA) and stimulated *ex vivo* with adenosine diphosphate (ADP; 7.8 and 125 µM; Sigma-Aldrich, The Netherlands), Thrombin Receptor Activator Peptide (TRAP6; 9.8 and 156 µM; Sigma-Aldrich) or cross-linked collagen-related-peptide (CRP-XL; 27.33ng/L and 655.7ng/L) and fixed after 20 minutes. CRP-XL was prepared as described previously[2]. Samples were directly measured on a FC500 (Beckman Coulter). Platelets were gated on size, granularity and CD61 positivity and the expression of CD62p and fibrinogen-binding on platelets were recorded using median-fluorescence intensity (MFI). A representative sample with gating strategy is shown in supplementary figure 1.

Platelet-leukocyte aggregation, monocyte subsets and T-cell activation were measured at baseline and after ten weeks in whole blood on a NAVIOS flow-cytometry (Beckman Coulter) as previously described[3]. Platelet-leukocyte aggregation was determined as the percentage of CD41 positive cells of specific leukocyte ($CD45^+$) populations (monocytes: $CD14^+$, CD4-cells: $CD3^+CD4^+$, CD8-cells: $CD3^+CD8^+$, B-cells: $CD19^+$ and NK-cells: $CD3^-CD56^+$). T-cell activation was determined as the percentage of CD4 or CD8-cells double positive for CD38 and HLA-DR. Monocyte subsets were determined as classical ($CD14^+CD16^-$), intermediate ($CD14^+CD16^+$) and non-classical ($CD14^{dim}CD16^+$) monocytes. Scattergrams of a representative sample with gating strategy is shown in supplementary figure 2. Soluble plasma proteins and thrombin-antithrombin (TAT) complexes, a marker for plasmatic coagulation, were measured in citrated platelet-poor-plasma (1500g centrifugation without brake, 10min, 20°C). Plasma concentrations of platelet factor 4 (PF4), beta-thromboglobulin (beta-tg/CXCL7), high-sensitive C-reactive protein (hsCRP, all R&D Systems) and TAT (Affinity Biologicals, USA) were measured using ELISA as previously described[4].

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Supplementary Table 1. Baseline characteristics.

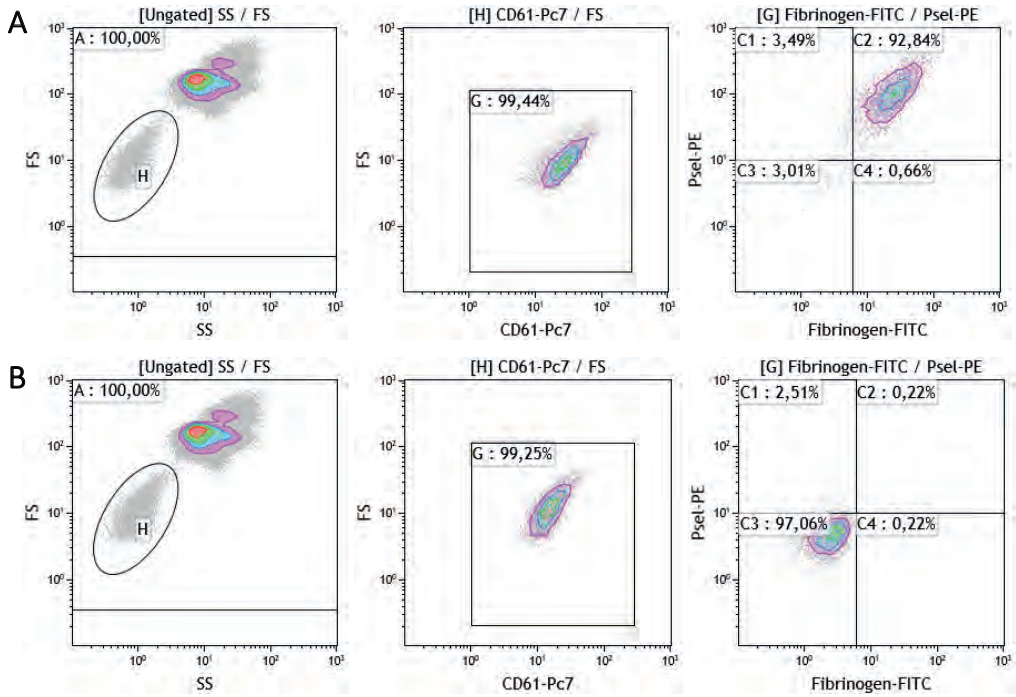
Patient characteristics	RAL (n=19)	CONT (n=21)	p-value
Age (years), median (IQR)	48 (43-54)	49 (42-61)	0.486
Female sex, n (%)	1 (5.2%)	0 (0%)	0.287
Body mass index, mean (SD) (kg / m²)	26.3 (±4)	24.7 (±3)	0.164
Current smoker, n (%)	4 (21.1%)	6 (28.5%)	0.583
Diabetes mellitus, n (%)	0 (0%)	2 (9.5%)	0.168
Hypertension, n (%)	3 (15.8%)	3 (14.3%)	0.894
Statin use, n (%)	2 (10.5%)	5 (23.8%)	0.270
cART regimens			
Backbone (2 NRTIs), n (%)			
TDF/FTC	13 (68.8%)	17 (81%)	0.736
ABC/3TC	5 (26.3%)	3 (14.3%)	0.342
TDF/3TC	1(5.2%)	0 (0%)	0.287
3TC/FTC	0 (0%)	1 (4.8%)	0.335
Boosted PI, n (%)	3 (15.8%)	3 (14.3%)	0.894
NNRTI, n (%)	15 (79%)	18(85.7%)	0.574
Zidovudine, n (%)	1 (5.2%)	0 (0%)	0.287
Viral load(<50 copies/mL), n (%)	19 (100%)	21 (100%)	NA
Nadir CD4 (10^6 cells/L), median (IQR)	290 (250-355)	280 (185-335)	0.707
CD4 count (10^6 cells/L), median (IQR)	760 (585-925)	650 (540-740)	0.175
eGFR, mean (SD) (ml/min /1.73m²)	81 (±12)	82 (±10)	0.668
HIV1 diagnosis (years), median (IQR)	10 (6-12)	9 (5-13)	0.333
cART exposure (years), median (IQR)	7 (5-10,5)	6 (5-11)	0.452

Data were analysed using independent T-test or Mann-Whitney-U test depending on distribution and no significant differences were observed defined as p-value <0.05. Interquartile range (IQR); standard deviation (sd); Estimated glomerular filtration rate (eGFR); Combination antiretroviral therapy (cART); nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs); tenofovir difumarate (TDF); emciterabine (FTC); abacavir (ABC); lamivudine (3TC); non-nucleoside reverse transcriptase inhibitor (NNRTI); boosted protease inhibitor (PI)

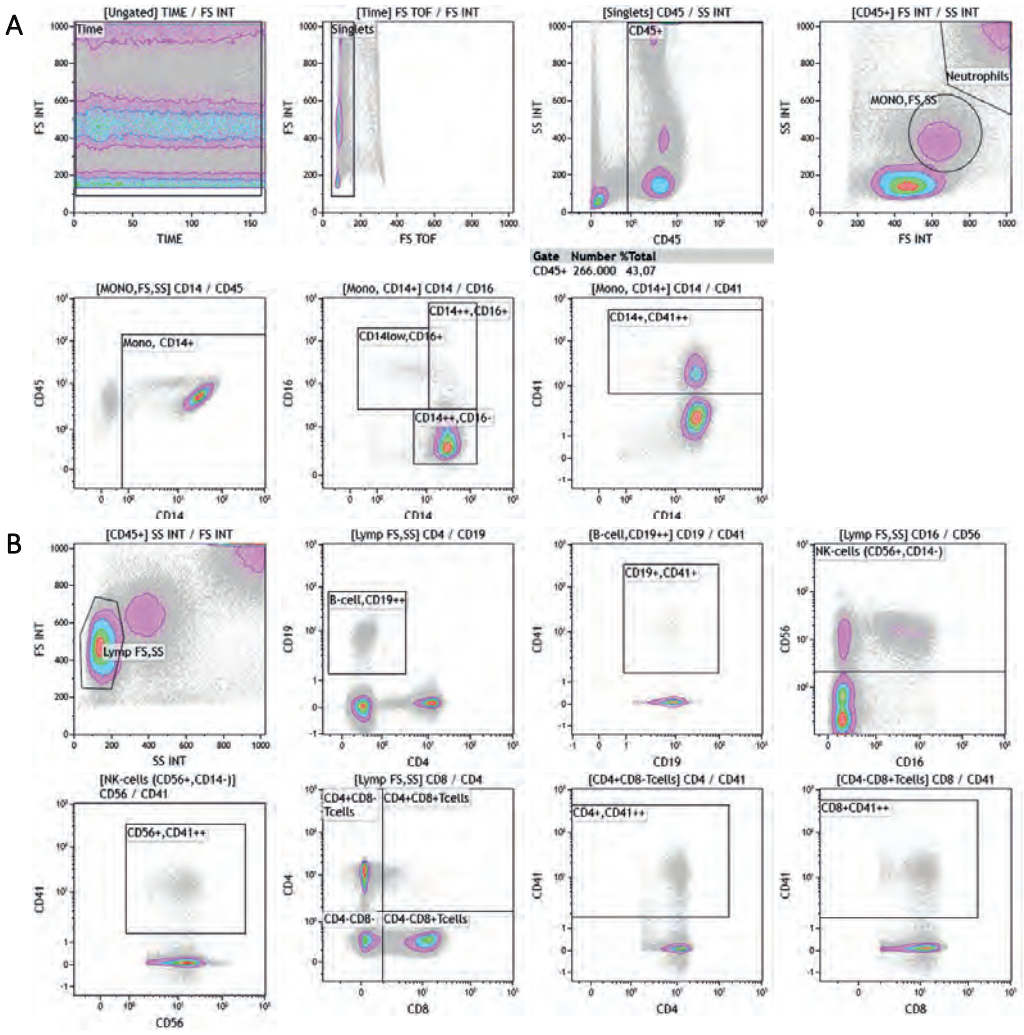
Supplementary Table 2. Platelet reactivity at baseline comparing subjects on abacavir vs tenofovir.

	P-selectin expression						
	Abacavir (n=8)			Tenofovir (n=32)			P-value
Stimulus	25%	median	75%	25%	median	75%	
ADP high	33.1	37.2	43.44	33.3	41.3	45.5	0.62
ADP low	27.7	34.5	38.5	29.9	36.9	42.8	0.41
CRP-XL high	81.7	87.3	97	75	83.4	90.3	0.37
CRP-XL low	34.5	43.9	61.4	29.1	61.2	73.6	0.41
TRAP high	7.2	46.4	57.2	8.57	31.2	60.59	0.72
TRAP low	64.2	73.5	77.9	70.7	74.1	81.4	0.55
unstimulated	3.65	4.72	5.12	3.75	4.45	5.2	0.97
	Fibrinogen binding						
	Abacavir (n=8)			Tenofovir (n=32)			P-value
Stimulus	25%	median	75%	25%	median	75%	
ADP high	8.57	13.6	17.2	11.1	13.5	20.5	0.55
ADP low	6.34	13.7	15.9	10.3	14.6	17.7	0.41
CRP-XL high	15.3	18.4	20.5	16.5	19.9	21.4	0.45
CRP-XL low	7.53	9.62	15	8.5	12.8	17.1	0.32
TRAP high	3.43	6.42	12.3	3.44	6.84	10.7	0.89
TRAP low	7.92	12.8	16.1	9.46	12	15.1	0.84
unstimulated	2.11	2.94	3.22	2.22	2.52	3.44	0.81

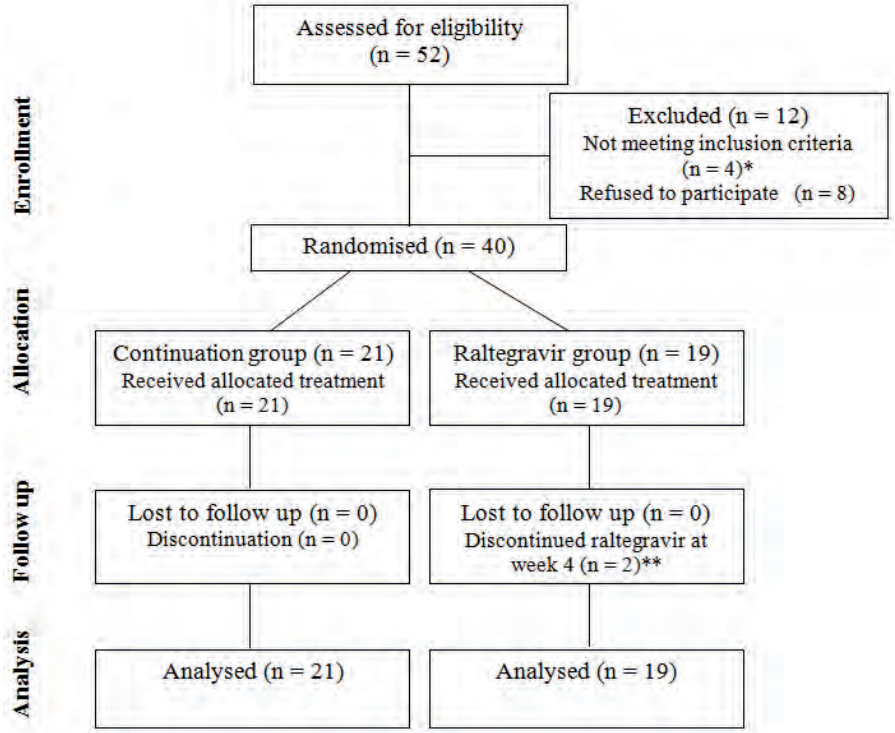
Platelet reactivity was determined by measuring platelet P-selectin expression and fibrinogen binding in unstimulated samples and after ex vivo stimulation with three agonists at two concentrations. Adenosine diphosphate (ADP; low: 7.8 µM and high 125 µM), Thrombin Receptor Activator for Peptide 6 (TRAP6; low: 9.8 µM and high: 156 µM) or cross-linked collagen-related-peptide (CRP-XL; low: 27.33ng/L or high: 655.7ng/L). Mann-Whitney U test was used for statistical analysis. No significant differences were observed defined as p-value <0.05.



Supplementary figure 1. Representative scatter gram with gating strategy of platelet reactivity. Platelet are selected based on side scatter (SS), forward scatter (FS) and CD61-positivity. Afterwards the median fluorescence intensity (MFI) is calculated of Fibrinogen and P-selectin to determine platelet reactivity. **A**) unstimulated sample. **B**) TRAP-stimulated sample. A minimum of 6000 platelets (CD61+) were analysed per condition.



Supplementary figure 2. Representative scatter grams and gating strategy for platelet-leukocyte aggregates. Gating strategy for monocyte subsets and platelet-monocyte aggregates (**A**) are shown in the upper graphs. Monocytes are gated based on side scatter (SS), forward scatter (FS) and CD14-positivity. Platelet-monocyte interaction is the the percentage of CD14+cells, positive for CD41. Gating strategy for lymphocyte subsets (**B**) (CD4, CD8, CD56 and CD19) are shown in the lower panels. Platelet lymphocyte aggregates are the percentage of cells positive for CD41. A minimum of 125.000 leukocytes (CD45+) were used for analysis.



Supplementary figure 3. Screening and enrolment flow diagram (CONSORT). 38 subjects completed the total follow-up period of 10 weeks. *Use of platelet inhibitor (aspirin), previous use of RAL **Two subjects discontinued raltegravir at week 4 because of grade I adverse events (mild sleeplessness and headache). In these subjects a measurement at week 4 was included as the end of study (EoS/week 10) for the primary outcome.

Chapter 8

Platelet microparticles inhibit IL-17 production by regulatory T cells through P-selectin

Sip Dinkla, Bram van Cranenbroek#, Wouter van der Heijden#, Xuehui He, Rike Wallbrecher, Ingrid Dumitriu, André van der Ven, Giel Bosman, Hans Koenen, Irma Joosten

#Equal contribution

Blood 2016; 127, 1976-1986, doi:10.1182/blood-2015-04-640300.

Abstract

Self-tolerance and immune homeostasis are orchestrated by FOXP3$^+$ regulatory T cells (Tregs). Recent data have revealed that upon stimulation, Tregs may exhibit plasticity toward a proinflammatory phenotype, producing interleukin 17 (IL-17) and/or interferon γ (IFN-γ). Such deregulation of Tregs may contribute to the perpetuation of inflammatory processes, including graft-versus-host disease. Thus, it is important to identify immunomodulatory factors influencing Treg stability. Platelet-derived microparticles (PMPs) are involved in hemostasis and vascular health and have recently been shown to be intimately involved in (pathogenic) immune responses. Therefore, we investigated whether PMPs have the ability to affect Treg plasticity. PMPs were cocultured with healthy donor peripheral blood–derived Tregs that were stimulated with anti-CD3/CD28 monoclonal antibodies in the presence of IL-2, IL-15, and IL-1β. PMPs prevented the differentiation of peripheral blood-derived Tregs into IL-17- and IFN-γ-producing cells, even in the presence of the IL-17-driving proinflammatory cytokine IL-1β. The mechanism of action by which PMPs prevent Treg plasticity consisted of rapid and selective P-selectin–dependent binding of PMPs to aCCR6$^+$HLA-DR$^+$memory-like Treg subset and their ability to inhibit Treg proliferation, in part through CXCR3 engagement. The findings that 8% of Tregs in the circulation of healthy individuals are CD41$^+$P-selectin$^+$ and that distinct binding of patient plasma PMPs to Tregs was observed support in vivo relevance. These findings open the exciting possibility that PMPs actively regulate the immune response at sites of (vascular) inflammation, where they are known to accumulate and interact with leukocytes, consolidating the (vascular) healing process.

Introduction

Although platelets are well known to be critical for hemostasis, it is becoming increasingly clear that platelets can actively modulate immune responses[1,2]. Upon activation, platelets release chemokines,

cytokines[1], and plasma membrane platelet-derived microparticles (PMPs) into the circulation[3]. PMPs are in the submicron range, are involved in hemostasis, and expose the lipid phosphatidylserine and molecules specific for platelets[3]. They have the ability to transfer membrane receptors[4] and microRNA[5] to other cells and play a role in the regulation of immunity[3,6].

Forkhead box P3 (FOXP3)$^+$ regulatory T cells (Tregs) are fundamental for immune homeostasis. Naturally occurring FOXP3$^+$, CD25^{high}, and CD127$^{low/-}$ Tregs comprise ;5% of the human peripheral blood CD4$^+$ T cells[7]. FOXP3 gene hyper demethylation is key to high and constitutive FOXP3 expression, which in turn is crucial for Treg suppressor function[8] and Treg stability[7]. We and others have shown that upon activation, CD4$^+$CD25^{high}FOXP3$^+$ Tregs can lose FOXP3 and differentiate into interleukin 17 (IL-17)- and interferon γ (IFN-γ)-producing cells[9-13]. Although proinflammatory cytokine-producing Tregs may reveal reduced suppressive capacity, a subset of IL-17-producing cells was shown to retain both FOXP3 expression and suppressive function[9,11,14,15]. Of note, Wang et al showed that in rheumatoid arthritis patients, peripheral blood IL-17-producing Tregs were suppressive, whereas their synovial fluid counterparts lost suppressive capacity[15]. The notion that this plasticity might have clinical implications was fuelled by a mouse study revealing that cytokine-producing Tregs induced autoimmunity[16]. In humans, increased numbers of proinflammatory cytokine-producing Tregs were found in patients with multiple sclerosis[17], Crohn disease[18], and psoriasis[19], where we identified IL-17-producing Tregs in the inflamed skin[19].

The ability of PMPs to influence cells of the immune system under inflammatory conditions that can also affect Treg phenotype led us to investigate the direct effect of PMPs in Tregs. Here, we show that PMPs inhibit the differentiation of highly purified human peripheral blood Tregs into IL-17- and IFN-γ–producing cells. The binding of PMPs to Tregs, and the subsequent inhibition of Treg differentiation, appeared to be P-selectin and partly CXCR3 dependent. Prior to and during Treg activation and subsequent differentiation, PMPs selectively bound a specific subset of memory-like Tregs expressing CCR6 and HLA-DR, known to bring forth IL-17-producing cells[10,20]. In the circulation, we found that;8% of Tregs are CD41 and P-selectin double positive. The finding that PMPs affect Treg proliferation provides a possible mechanism by which PMP binding to the memory-like Treg subset affects their IL-17-producing capacity. These findings open the exciting possibility that PMPs

actively regulate the immune response at sites of (vascular) injury, where PMPs are known to accumulate[21].

Methods

HIV-infected adults, acute coronary syndrome patients, and healthy volunteers

Peripheral blood (EDTA) was obtained from patients and healthy volunteers. HIV-infected adults on stable combination antiretroviral therapy with undetectable viral load (<50 copies/mL) and CD4 counts <300 cells/mm3 were eligible for enrolment. Exclusion criteria were the use of platelet inhibitors or an active concomitant infection (e.g. hepatitis C or B). The study protocol was approved by the medical ethical committee Arnhem-Nijmegen (Radboud University Medical Center). Patients with a diagnosis of acute coronary syndrome (ACS; ST-elevation myocardial infarction and non–ST-elevation myocardial infarction). Blood samples were collected 4 to 12 hours from the start of chest pain. Patients with evidence of infectious diseases, malignancies, hematologic or immunologic disorders, treatment with anti-inflammatory drugs other than aspirin, and an ejection fraction ,40 were excluded. The study was approved by the local research ethics committee (St. George's, University of London). Both studies adhered to the Declaration of Helsinki. Signed informed consent was obtained from all patients and healthy volunteers.

PMP isolation, storage, and anti–P-selectin monoclonal antibody neutralization

Single donor platelet-rich plasma (PRP) was collected from 3 healthy donors by apheresis upon written informed consent according to the Declaration of Helsinki. Using a component collection system (MCS1; Haemonetics, Braintree,MA) in combination with acid citrate dextrose solutionA, 3003109 leukoreduced (<1 X 10^6 leukocytes/U) platelets were collected in 300 mL plasma and stored in a lateral shaking device at 22°C 6 2°C for 7 days. At day 7 of storage (the maximum storage period in The Netherlands), PMPs were isolated from the PRP product under sterile conditions using differential centrifugation as previously described[22]. The PRP was centrifuged at 2000g for 15 minutes to remove platelets and cell debris. The supernatant was aliquoted, centrifuged for 45 minutes at 20 000g to pellet PMPs, and washed with 0.22mm filtered calcium-free Ringer's solution (125mM NaCl, 5mM KCl, 1mM MgSO4, 32mM HEPES, 5mM glucose, and 0.2% bovine serum albumin [pH 7.4]). The PMPs were resuspended in calcium-free Ringer's solution, snapfrozen with liquid N_2, and stored at 280°C. PMP purity/quantity was assessed using a Gallios flow cytometer (Beckman Coulter, Brea, CA) as described in supplemental Methods. PMP-associated P-selectin neutralization and washed platelet isolation, activation, and subsequent PMP generation are described in the supplemental Methods. Plasma derived microparticle isolation is described in the supplemental Methods.

Cell isolation, culture, and anti-CXCR3 and anti-CD40 mAb neutralization

Peripheral blood mononuclear cells (PBMCs) were isolated by density-gradient centrifugation (Lymphoprep; Nycomed-Pharma AS, Oslo, Norway) of buffy coats obtained from healthy donors upon written informed consent according to the Declaration of Helsinki. $CD4^+$ T cells were purified from PBMCs by magnetic bead sorting using CD4 MicroBeads (Miltenyi Biotec, Leiden, The Netherlands) according to the manufacturer's instructions. Purified $CD4^+$ T cells were labelled with anti-CD4–fluorescein isothiocyanate (FITC) (MT310; DAKO, Glostrup, Denmark) and anti-CD25-PE (MA251; BD Biosciences, Erembodegem, Belgium) monoclonal antibodies (mAbs), after which $CD4^+CD25^{high}$ cells (Tregs) were isolated by high-purity (>99%) flowcytometric cell sorting using an Aria cell sorter (Beckman Coulter).

Cells (2.5 x 10^4/well) were cultured in culture medium (RPMI-1640 with Glutamax supplemented with 0.02 mM pyruvate, 100 U/mL penicillin, 100mg/mL streptomycin [Gibco, Paisley, United Kingdom], and 10% human pooled serum) at 37°C, 95% humidity, and 5% CO2 in 96-well round-bottom plates (Greiner, Frickenhausen, Germany). Anti-CD3/anti-CD28 mAb-coated beads (1:10; Invitrogen, Breda, The Netherlands) and recombinant human cytokines IL-2 (25 U/mL; Cetus, Amsterdam, The Netherlands), IL-15 (10 ng/mL), and IL-1β (50 ng/mL) (Biosource, Etten-Leur, The Netherlands) were added at the start of the cultures, in the presence or absence of PMPs. The suppression assay, transwell assay, and CD40 and CXCR3 neutralization are described in supplemental Methods.

Flow cytometry and antibodies

Cells were phenotypically characterized using the Navios flow cytometer (Beckman Coulter) and conjugated mAbs supplied in supplemental Methods. In order to study cell proliferation, Pacific Blue succinimidyl ester (Invitrogen) labelling (0.012 mg/mL) of Tregs prior to culture was performed. Intracellular analysis was performed after fixation and permeabilization using Fixation/Permeabilization reagent (eBioscience, Uithoorn, The Netherlands). Before intracellular cytokine measurements, the cells were stimulated for 4 hours with phorbol 12-myristate 13-acetate (PMA;12.5 ng/mL) plus ionomycin (500 ng/mL) in the presence of Brefeldin A (5 mg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands). Peripheral blood staining is described in supplemental Methods. All flow cytometry data were analyzed using Kaluza software (Beckman Coulter). Proliferation Platform of FlowJo 7.6 was employed to assess proliferation.

Confocal laser scanning microscopy and FOXP3 gene methylation

See supplemental Methods.

Statistics

Repeated-measures 1-way (plus Tukey's post-test) or 2-way (plus Bonferroni post-test) ANOVAs were used for statistical analysis. The reported (2-sided) P values of <.05 were considered significant and are indicated with an asterisk(*). Data in the text are presented as mean 6 ± standard deviation.

Results

PMPs inhibit CD25highFOXP31 Treg differentiation into IL-17–producing cells

We reasoned that PMPs, with their arsenal of immune-mediating molecules[3], and their ability to interact with cells of the immune system[6,23], might affect the differentiation of Treg into IL-17–producing T cells[9]. To obtain large amounts of PMPs, we used PMPs isolated from 7-day-stored platelet apheresis products of healthy donors, known to accumulate PMPs during storage[24]. PMP purity, size, and number were assessed by flow cytometry, based on exposure of phosphatidylserine (PS) and CD41 (Figure1A and supplemental Figure 1).Over 99% of all measured events were smaller than 1 µm in diameter, and more than 90% stained positive for CD41 and PS. Stored platelets and their PMPs were further characterized for exposure of PS, CD40, CD41, and activation markers P-selectin and CD63 (supplemental Figure 2).

PMPs were then cocultured with highly purified $CD4^+CD25^{high}CD27^+CD127^{low/-}FOXP3^+$ Treg isolated from PBMCs by fluorescence-activated cell sorting (supplemental Figure 3). Typically, anti-CD3/CD28 mAb stimulation of this population in the presence of IL-2, IL-15, and IL-1β leads to a substantial number of IL-17–producing cells, with concomitant loss of FOXP3[9].

The addition of PMPs to the freshly isolated Tregs at ratios of 2.4,12, or 60 PMPs per cell inhibited the induction of IL-17 and IFN-γ production in a dose-dependent manner (Figure 1B-C,E-F). Similarly, the loss of FOXP3 expression was inhibited (Figure 1D-F), and a higher level of CpG demethylation in the Treg-specific demethylation region of theFOXP3genewas detected (supplemental Figure 4). Interestingly, of the few cells that managed to acquire IL-17–producing capacity in the presence of PMPs, most still expressed FOXP3 (Figure 1D), suggesting that the differentiation process was partially hampered. Furthermore, PMP binding was higher in $IL\text{-}17^+FOXP3^+$ cells than in $IL\text{-}17^+FOXP3^-$ cells (supplemental Figure 5). Previous studies have revealed that $CD4^+CD25^+$ Tregs expressing CD27 and/or CD62L constitute highly suppressive Treg subsets[25,26]. Similar to the expression of FOXP3, loss of CD27 and CD62L was also prevented, whereas PMPs inhibited the percentage of cells that acquired the TH17[27] and memory-associated[28] chemokine receptor CCR6 during culture (Figure 1E-F). PMPs isolated from 2-day–stored platelet apheresis products also inhibited the generation of IL-17–producing cells (supplemental Figure 6), indicating that there are no major qualitative storage period–associated differences in this regard.

To examine whether the effects of PMPs on Treg differentiation are stored-platelet specific or can be attributed to other sources and causes of PMP production, we used freshly isolated washed platelets activated with thrombin receptor activator peptide 6 (TRAP) in the presence of calcium and shear stress for optimal PMP generation. The PMPs formed by these TRAP-activated washed platelets had similar effects on Treg differentiation as PMPs isolated from stored platelet units, including reduced induction of IL-17 and IFN-γ production (supplemental Figure 7). These data underscore the potential of both platelet apheresis unit–derived, as well as activated platelet-derived, PMPs to promote Treg stability. In addition, we also observed a reduction in IL-17 production when culturing Tregs in the presence of TRAP-activated washed platelets themselves (supplemental Figure 8).

P-selectin and CXCR3 mediate PMP inhibitory effects on Treg differentiation

Subsequently, the question arose which molecular component(s) of the PMPs were responsible for the inhibitory effect observed. Using a transwell setup with intracellular IL-17 expression as a readout (Figure 2A), we observed that Treg differentiation was contact dependent. This ruled out the possibility of cytokine scavenging or release by the PMPs significantly affecting Treg differentiation.

As we observed clear staining for the platelet-specific marker CD41 on Tregs cocultured with PMPs (Figure 2B), even after repeated washing steps, we proposed that PMPs firmly bind to these cells and postulated that adhesion molecules, several of which are present on the PMP membrane[3], might be involved in this PMP–Treg contact-dependent interaction. Upon activation, platelets typically expose the adhesion molecule P-selectin, which they can pass onto the PMPs they generate[29]. Like their parental cells, PMPs are able to bind immune cells expressing the P-selectin ligand P-selectin glycoprotein ligand-1 (PSGL-1)[30]. This led us to investigate whether P-selectin is involved in the observed PMP adhesion to Tregs and/or the inhibition of their differentiation.

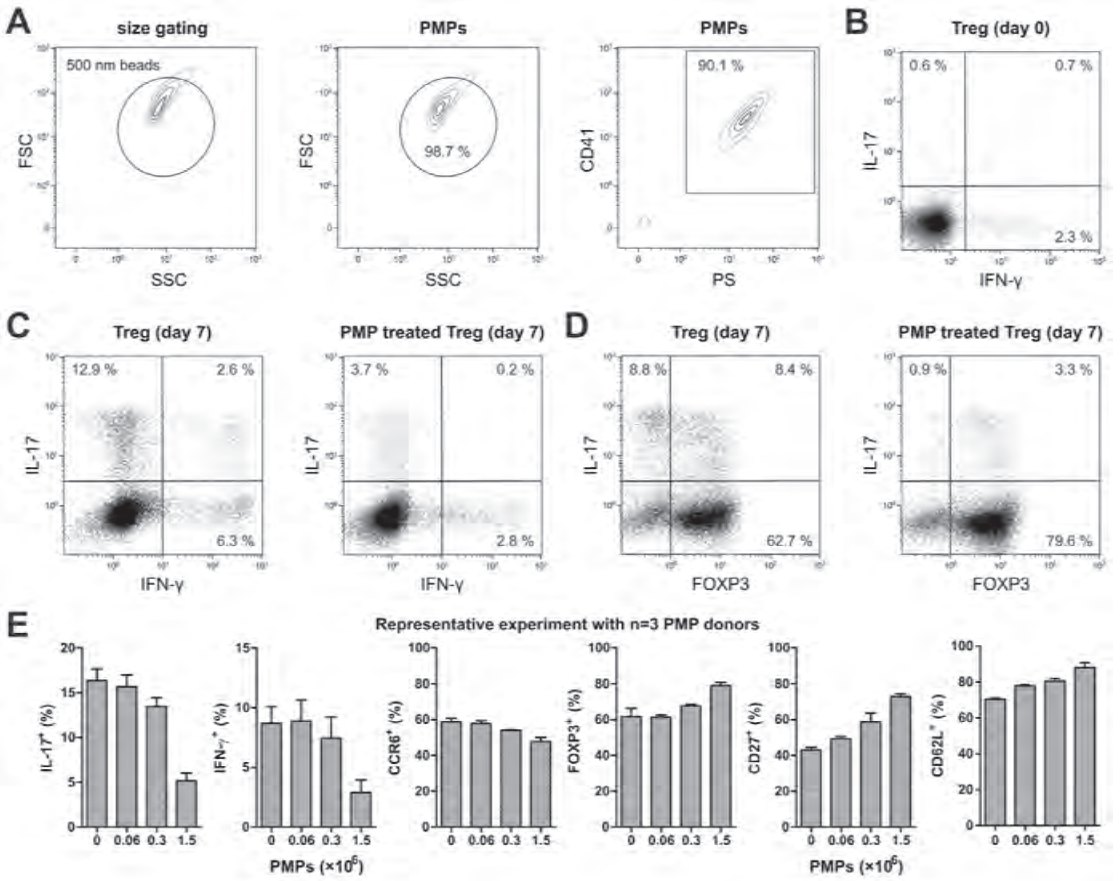
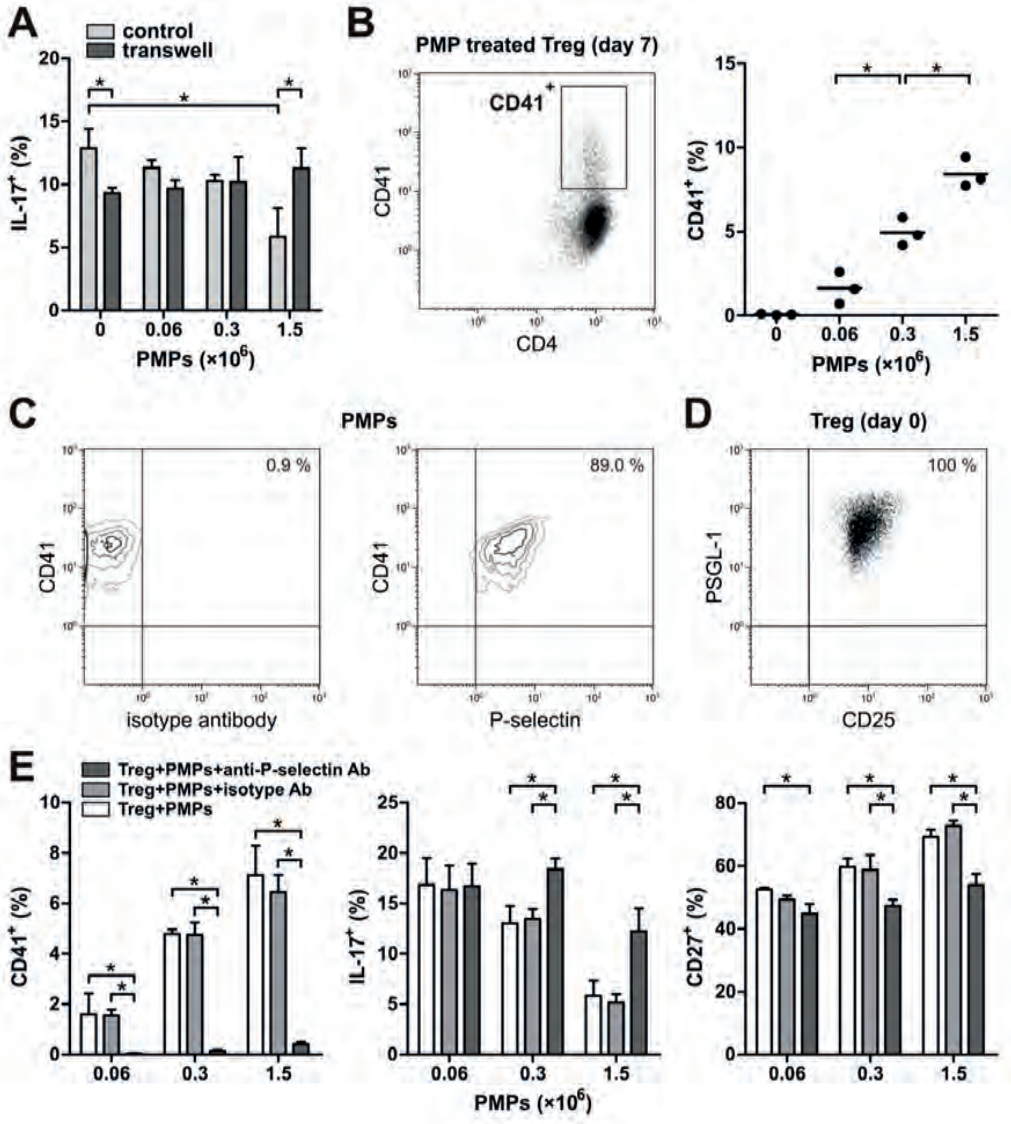


Figure 1. PMPs inhibit Treg differentiation into IL-17-producing cells. Treg were isolated from healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads and recombinant human IL-2, IL-15, and IL-1β in the presence or absence of allogeneic PMPs isolated from platelet apheresis units of three healthy donors. Flow cytometry analyses of intracellular cytokines were performed after stimulation with PMA and ionomycin, in the presence of Brefeldin A. (**A**) CD41+PS+ PMPs <1 μm were purified from platelet apheresis units of healthy donors by differential centrifugation and analyzed by flow cytometry for phosphatidylserine (PS) and CD41 exposure as described in Methods. Size beads (500 nm) were used to set a size exclusion gate, and fluorescent count beads (~10 μm) were added for quantification. The contour plots are representative for six donors. (**B**) Representative experiment showing intracellular IL-17 and IFN-γ staining of the Treg at day 0. (**C-D**) Representative example of intracellular IFN-γ, IL-17 and FOXP3 expression by Treg that were cultured for 7 days with and without 1.5×106 PMPs. (**E**) A representative experiment performed with Treg from a single donor and increasing PMP concentrations from three different healthy donors. Surface CCR6, CD27, CD62L and intracellular IL-17, IFN-γ, and FOXP3 staining of the Treg at day 7 of culture are shown. Error bars represent SD. (**F**) Cumulative data of different Treg donors (n=3-5) showing surface CCR6, CD27, CD62L, and intracellular IL-17, IFN-γ, and FOXP3 staining of Treg cultured for 7 days with increasing PMP concentrations. Each donor's Tregs are shown as a dot representing the mean response of those Tregs to the three different donor's PMPs. The mean is presented as bars. *P<0.05



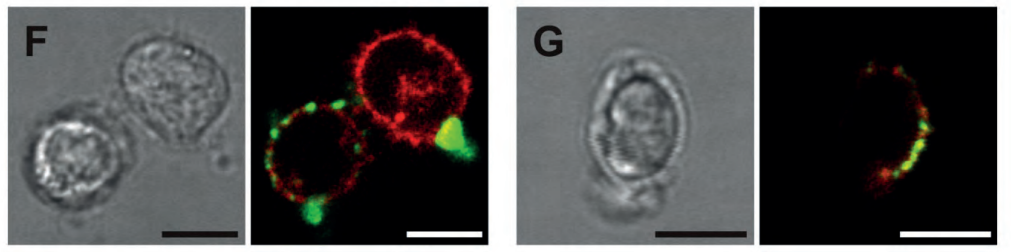


Figure 2. PMP inhibition of Treg differentiation is P-selectin-dependent. Treg were isolated from healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads and recombinant human IL-2, IL-15, and IL-1β in the presence or absence of allogeneic PMPs isolated from platelet apheresis units of three healthy donors. Flow cytometry analyses of intracellular cytokines were performed after stimulation with PMA and ionomycin, in the presence of Brefeldin A. (**A**) Intracellular IL-17 expression in Treg that were co-cultured with PMPs as a standard mixed culture (control), or separated by a porous membrane (transwell). (**B**) The presence of the platelet-specific marker CD41 was assessed on the $CD4^+$ Treg after co-culture with increasing PMP concentrations. Each donor's Tregs are shown as a dot representing the mean response of those Tregs to the three different donor's PMPs. The mean is presented as bars. *P<0.05. (**C**) Representative contour plots of PMPs stained with anti-CD41-PE and anti-P-selectin-FITC or isotype mAbs. The contour plots are representative for three donors. (**D**) Treg expression of PSGL-1 and CD25 at day 0 of culture. (**E**) Surface CD41, CD27 and intracellular expression of IL-17 on Treg co-cultured with untreated, isotype, or anti-P-selectin mAb-neutralized PMPs. The graphs are representative for two Treg donors. Mean and SD are shown. *P<0.05. (**F**) Confocal laser scanning microscopy of Treg co-cultured with 1.5×10^6 PMPs for 16 hours in the presence of anti-CD3/anti-CD28-coated-beads and recombinant human IL-2 and stained for CD41 (FITC, green) and CD25 (APC, red), or (**G**) P-selectin (FITC, green) and PSGL-1 (APC, red). Scale bar = 5 µm. Representative images are shown. Images were acquired with a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with a HCX-PL-APO 63×1.2 water immersion lens while cells were maintained at 37°C in HBS buffer. Leica LAS AF acquisition software was used.

First, we established that the majority of PMPs (86.3% 6 2.4%) indeed expressed P-selectin (Figure 2C) and that Tregs expressed P-selectin ligand PSGL-1 at day 0 (Figure 2D). Subsequently, we performed neutralization experiments. Prior to coculture with Tregs, PMPs were incubated with an FITC-conjugated antagonistic anti–P-selectin mAb, an isotype control mAb, or in buffer only and subsequently washed. Although isotype mAb did not bind to the PMPs (0.4 6 0.1%), PMPs were effectively bound by the anti–P-selectin mAb (80.6%64.4%). The anti–P-selectin mAb was still present on the PMPs at day 7 of culture with Treg (77.4% 6 7.1%). Notably, the neutralization of P-selectin not only abrogated (CD411) PMP adhesion to the Treg (Figure 2E) but also greatly blunted their inhibitory effect on Treg differentiation into IL-17–producing cells and loss of the Treg marker CD27 (Figure 2E). Neutralization of P-selectin also restored the loss of FOXP3 and CD62L and the increase in IFN-γ expression by Tregs during culture (supplemental Figure 9).

Confocal laser scanning microscopy of Tregs cocultured with PMPs confirmed the binding of $CD41^+$PMPs to the cell membrane of part of the Treg (Figure 2F). The majority of these PMPs had a diameter of 0.5 mm or less, whereas some were larger PMPs and/or PMP aggregates. In addition, adherent P-$selectin^+$ PMPs colocalized with PSGL-1 on the Treg membrane (Figure 2G).

Then, we focused attention on platelet-associated immune signalling molecules. Because CD40-CD40L costimulation has been implicated in PMP-mediated signaling[23], we investigated the involvement of this pathway. However, neutralization of CD40 in culture with an antagonistic anti-CD40 mAb did not reduce the inhibitory effect of PMPs on Tregs (supplemental Figure 10).

Platelet factor 4 (PF4/CXCL4), predominantly expressed by platelets, is a CXCR3 ligand and triggers CXCR3-mediated signalling in activated T cells[31] leading to inhibition of proliferation and IFN-γ secretion[32]. Moreover, PF4 was found to be a negative regulator of Th17 differentiation[33]. We therefore assessed CXCR3 expression on Tregs during culture and performed CXCR3 neutralization experiments. CXCR3 expression increased upon Treg activation (Figure 3A-C), and CXCR3 neutralization using an antagonistic CXCR3 mAb reduced the inhibitory effect of PMPs on the generation of IL-17–producing cells (Figure 3D). These data suggest that CXCR3-signaling contributes to the observed effects of PMPs on Treg differentiation.

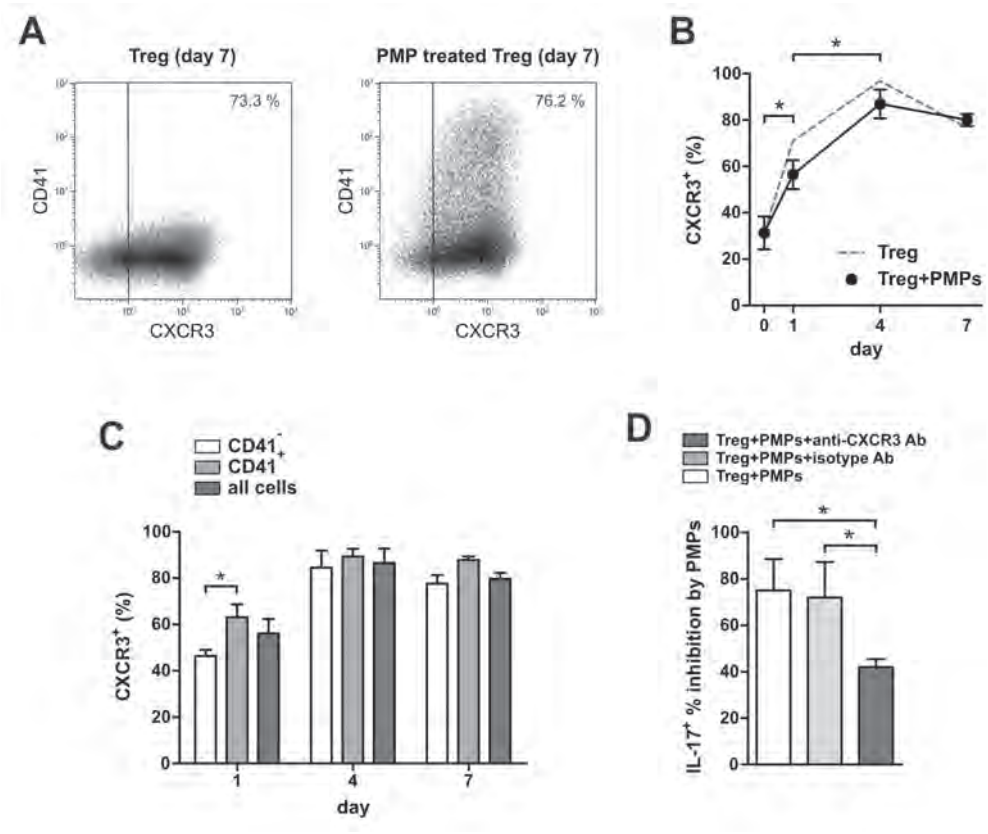


Figure 3. CXCR3 neutralization leads to partial loss of PMP-mediated inhibition of IL-17 production by Treg. Treg were isolated from healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads and recombinant human IL-2, IL-15, and IL-1β in the presence or absence of 1.5×106 allogeneic PMPs isolated from platelet apheresis units of three healthy donors. Flow cytometry analyses of intracellular cytokines were performed after stimulation with PMA and ionomycin, in the presence of Brefeldin A. (**A**) Example dot plots of day 7 CXCR3 expression on Treg. (**B**) CXCR3 expression on Treg throughout culture. (**C**) CXCR3+ Treg present in the CD41- and CD41+ subpopulations in time. (**D**) Day 7 intracellular staining of IL-17 on Treg cultured with or without PMPs in the absence or presence of isotype or antagonistic anti-CXCR3 mAb. The percent PMP-mediated inhibition of IL-17-expressing Treg is displayed in the graph. Results for three Treg donors are shown. Mean and SD are shown. *P<0.05.

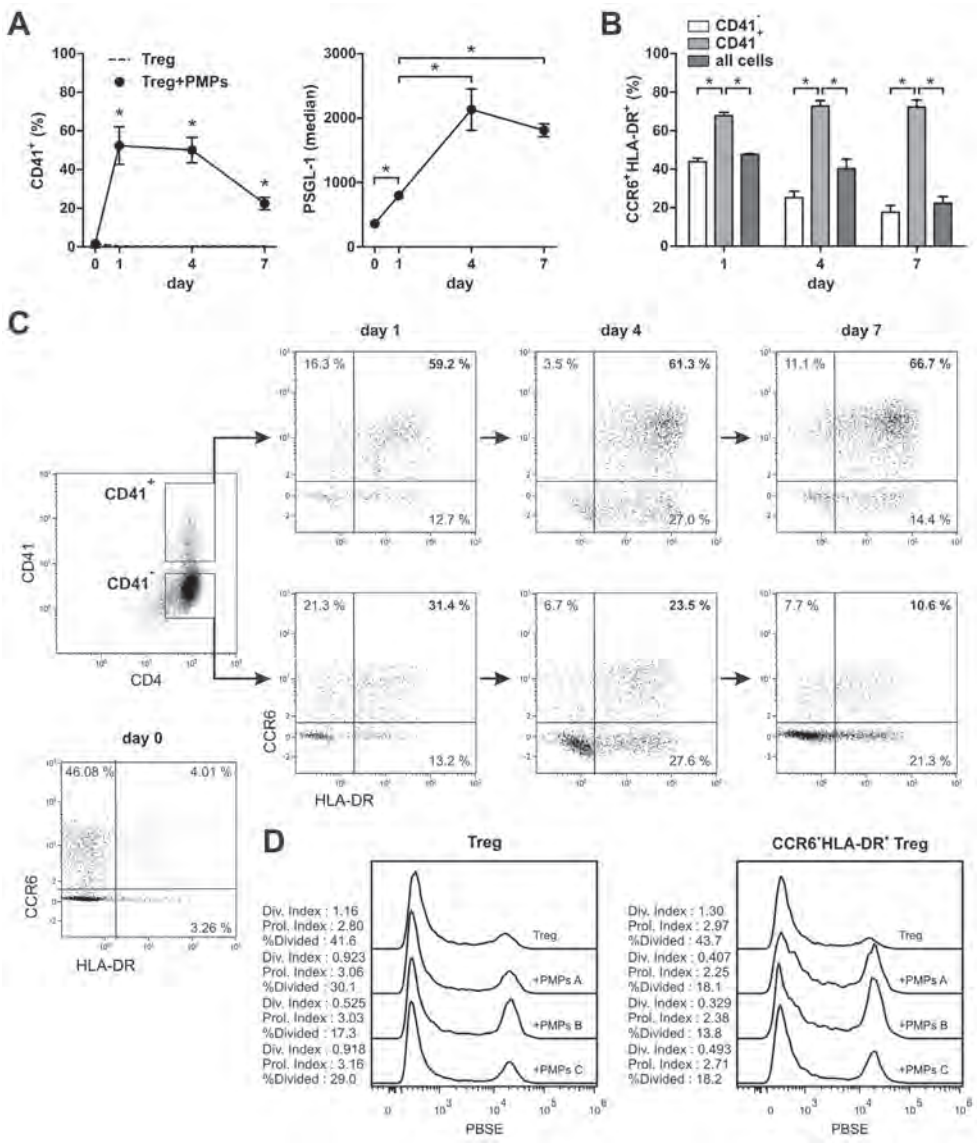


Figure 4. PMPs selectively target CCR6+HLA-DR+ Treg prior to, and during, activation. Treg were isolated from healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads, recombinant human IL-2, IL-15, IL-1β and 1.5×106 allogeneic PMPs isolated from platelet apheresis units of three healthy donors. Treg were stained for surface markers and analyzed by flow cytometry at day 0, 1, 4 and 7. (**A**) CD41+ PMP-bound Treg (n=5 Treg donors), and the expression level of PSGL-1 on the Treg (n=3 PMP donors). (**B**) CCR6+HLA-DR+ Treg present in the CD41- and CD41+ subpopulations. (**C**) Example CCR6/HLA-DR dot plots of PMP-negative (CD41) and PMP-positive (CD41+) Treg. Mean and SD for three PMP donors are shown. *P<0.05. Results representaive for five Treg donors are shown. (**D**) Day 7 cell proliferation of PBSE-labeled Treg co-cultured with and without PMPs was assessed. The division index, proliferation index and percentage of divided cells were determined for total Treg and CCR6+HLA-DR+ Treg using FlowJo 7.6. Results are shown for Treg cultured alone and Treg co-cultured with PMPs of three different PMP donors.

PMPs selectively target CCR61HLA-DR1 memory-like and IL-17–producing Tregs

Only a subset of peripheral blood–derived Tregs produces IL-17 upon stimulation[9-13]. Insight into the features of this subset might guide us in the characterization and regulation of these cells. Sparked by the observation that the PMPs only associated with a subset of the Tregs (Figure 2B), while inhibiting IL-17 production, we argued that we might use this characteristic to pinpoint the IL-17 precursors within the whole Treg population.

To establish the identity of the Treg subset(s) targeted by the PMPs, and to determine whether this interaction occurs before Treg differentiation progresses, we followed PMP binding to Treg in time during a 7-day culture (Figure 4). Binding of PMPs to Tregs already occurred within 1 day of culture (Figure 4A). Concomitantly, an increase in the expression of the P-selectin ligand PSGL-1 was observed (Figure 4A).

CCR6-positive Tregs constitute a memory-like phenotype28 that was found to harbor the majority of Tregs with potential IL-17–producing capacity[9,10,20]. Therefore, we assessed CD41 and CCR6 expression on the Tregs over time, combined with HLA-DR, which defines an activated Treg subset[12,34]. Strikingly, a strong binding preference of PMPs was observed for CCR6$^+$HLA-DR$^+$ double-positive, but not single-positive, Tregs throughout days 1 to 7 of culture (Figure 4B-C). IL-17–producing cells were positive for both CCR6 and HLA-DR and expressed the TH17-associated transcription factor RORγT (supplemental Figure 11). Further phenotyping of the CD41$^+$Treg revealed that the majority expressed the functional Treg marker CD39[35] and contained a higher proportion of CD161$^+$ cells (data not shown), associated with potential IL-17–producing capacity[36]. Coculture of Tregs with PMPs reduced Treg proliferation, particularly in CCR6$^+$HLA-DR$^+$Tregs (Figure 4D).

This provides a potential mechanism by which CCR6$^+$HLA-DR$^+$Treg differentiation into IL-17-producing cells is inhibited. Finally, sorted CCR6$^+$ and CCR6$^-$ Treg populations were cultured with and without PMPs (supplemental Figure 12). As expected, IL-17- and IFN-γ-producing Tregs were predominantly found in the CCR6$^+$ Treg population (supplemental Figure 12B). Coculture of CCR6$^+$ Treg with PMPs effectively inhibited the generation of IL-17- and IFN-γ-producing Tregs (supplemental Figure 12B), prevented the loss of FOXP3, and secured the suppressive capacity (supplemental Figure 12C-D).

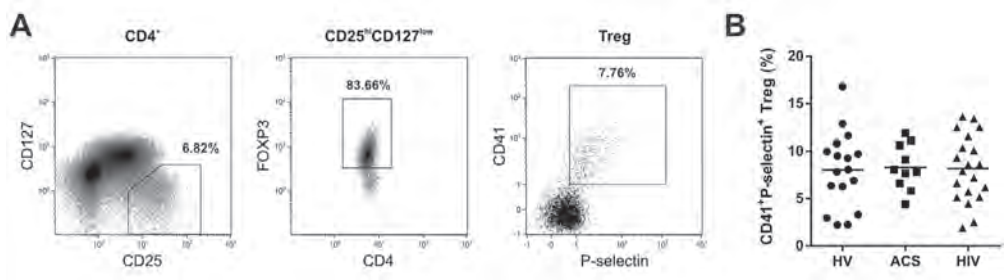


Figure 5. Detection of CD41⁺P-selectin⁺ Treg in peripheral blood of healthy volunteers, HIV-infected adults and patients with acute coronary syndrome. Flow cytometry analyses of peripheral blood obtained from patients with acute coronary syndrome (ACS, n=10), HIV-infected adults on stable combination antiretroviral therapy (n=20) and healthy volunteers (HV, n=17) for surface CD4, CD127, CD25, CD41, P-selectin and intracellular FOXP3 expression. (**A**) Representative example of $CD4^+CD25^{high}CD127^{low}FOXP3^+$ Treg gating and their exposure of CD41 and P-selectin in peripheral blood of a healthy volunteer. (**B**) Percentage of CD41 and P-selectin double positive Treg in peripheral blood of ACS patients, HIV-infected adults and healthy volunteers.

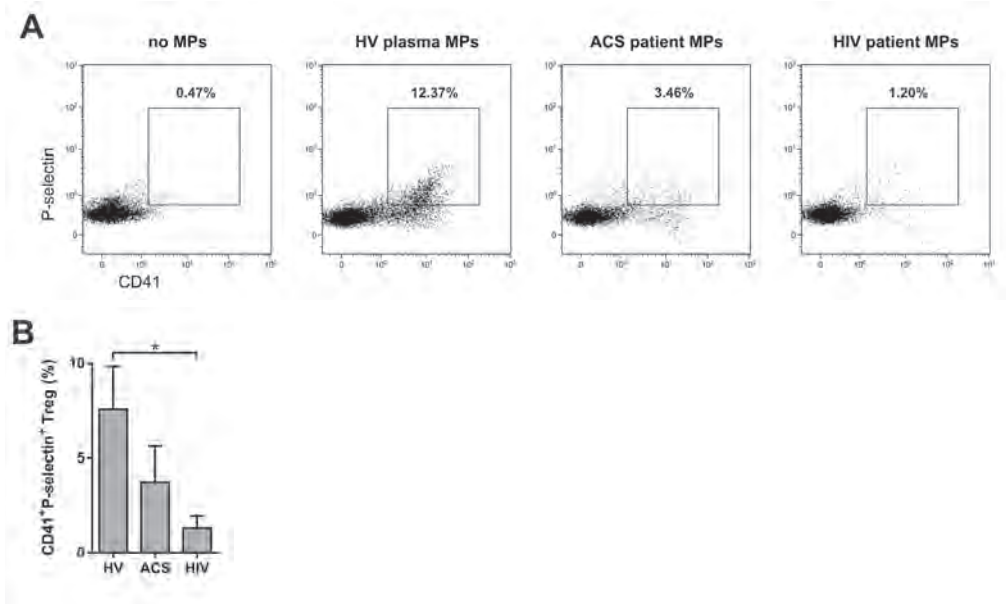


Figure 6. Reduced binding of plasma-derived PMPs from HIV-infected adults to Treg. Treg isolated from five healthy donors were cultured for 16 hours with recombinant human IL-2 in the presence or absence of total microparticle fractions isolated by differential centrifugation from platelet poor plasma of patients with acute coronary syndrome (ACS, n=3), HIV-infected adults on stable combination antiretroviral therapy (HIV, n=3) and healthy volunteers (HV, n=3). Treg were stained for surface CD4, CD25, CD41, P-selectin and intracellular FOXP3 and analyzed by flow cytometry. (**A**) Dot plots show the percentage of CD41 and P-selectin double positive $CD4^+CD25^{high}FOXP3^+$ Treg. The dot plots were generated using merged data of three plasma microparticle donors. (**B**) The percentage of CD41 and P-selectin double positive Treg (n=3 plasma microparticle donors). Representative results are shown. *P<0.05

Evidence for CD41⁺P-selectin⁺ Tregs in the circulation

To provide evidence for human in vivo relevance of Treg/PMP interaction, we investigated the presence of PMP-bound Tregs in the circulation. We established that ~8% of the peripheral blood Tregs from healthy individuals (n=17) were CD41 and P-selectin double positive (Figure 5). Subsequently, we examined PMP-Treg binding in patients known to exhibit enhanced platelet activation and PMP formation in the circulation[29,37-39], ie, HIV-infected adults on stable combination antiretroviral therapy (n=20) and patients with ACS (n=10).We confirmed higher numbers of (PS^+CD41^+P-selectin$^+$) PMPs in plasma of ACS patients ($1.17x10^7 \pm 0.93x10^7$/mL) and HIV-infected individuals ($6.2 \times 10^7 \pm 3.16 \times 10^7$/mL) compared with healthy volunteers ($0.35 \times 10^7 \pm 0.36 \times 10^7$/mL). Notwithstanding the higher patient PMP levels, we found similar percentages of PMP-bound Tregs in peripheral blood compared with healthy individuals (Figure 5B). However, we did observe significantly less binding of HIV patient plasma PMPs to healthy control Tregs (Figure 6), whereas binding of PMPs from healthy individuals to Tregs of HIV-infected adults was comparable to their binding to Tregs of healthy volunteers (supplemental Figure 13). This suggests altered PMP binding capacity in these patients.

Discussion

Tregs constitute an essential component in the maintenance of tissue homeostasis and the prevention of (auto)inflammatory processes. Dysregulation of Treg function is associated with a myriad of pathologies (e.g. psoriasis, multiple sclerosis, rheumatoid arthritis, and graft-versus-host disease)[7,8,40]. We and others have shown that Tregs may produce IFN-γ and IL-17 when stimulated under proinflammatory conditions[9-13] and that these differentiated cells are found at sites of inflammation[19,41].

In our search for immunomodulatory components that influence the Treg differentiation process, we hypothesized that PMPs might constitute a relevant force. Like their parental cells, PMPs are involved in hemostasis, maintenance of vascular health, and immunity[3,6,21,23,29,30,42].

Here, we report that PMPs have the capacity to prevent the differentiation of Tregs into IL-17- and IFN-γ-producing cells, even in the presence of proinflammatory cytokines. The mechanism of action consists of rapid and selective P-selectin-dependent binding of PMPs to a specialized $CCR6^+HLA\text{-}DR^+$ memory-like Treg subset that is known to contain the majority of progenitor Th17-like cells[10,20], disrupting their proliferation and eventual differentiation into IL-17 producers. The few Tregs with bound PMPs that did acquire IL-17-producing capacity, were in a $FOXP3^+IL\text{-}17^+$ transitory state, likely due to the inhibitory effect of the PMPs. In addition, we found evidence for PMP-bound human Tregs in the circulation, suggesting a physiological function of this process.

Leukocytes play a central role in wound repair and are recruited after coagulation and the resulting vascular inflammation have been initiated[43]. PSGL-1 is expressed on most leukocytes and mediates

their recruitment to inflamed endothelium by binding P- and E-selectin on activated endothelial cells[44]. P-selectin exposure by adherent activated platelets further assists leukocyte recruitment to the inflamed tissue[1]. Moreover, activated platelets release P-selectin$^+$PMPs[29], which also accumulate at sites of (vascular) injury[21], and are able to bind PSGL-1$^+$ leukocytes[30]. We show that PMPs can bind peripheral PSGL-1$^+$memory-like Tregs through P-selectin-mediated adhesion, possibly participating in their recruitment to sites of injury. Tregs were indeed found to be involved in local tissue repair by restricting the proinflammatory response in a lung injury mouse model[45]. In patients with systemic vasculitis, vascular infiltration of Th17 and Th1 cells was enhanced, and fewer Tregs were observed in the lesions[46]. Interestingly, FOXP3$^+$ cells were shown to contribute to the IL-17 production in vascular lesions of these patients[41]. Our observations raise the tantalizing possibility that PMPs might assist in the control and eventual resolution of inflammation during tissue repair by stabilizing the Treg phenotype and preventing their differentiation into Th1 or Th17-like cells in a proinflammatory microenvironment. One could argue that PMPs can easily enter inflamed tissue due to their small size and ability to interact with leukocytes, allowing them to hitchhike to the site of inflammation to exert their regulatory function. Increased PSGL-1 expression was observed on all Tregs, concomitantly with PMP binding upon Treg activation. Nevertheless, the PMPs showed a high preference for CCR6$^+$HLA-DR$^+$Tregs, suggesting additional requirements for effective PMP binding. A possible explanation for this differential binding is that glycosyltransferases required to modify PSGL-1 to its active binding state are upregulated only upon T-cell activation[47].

PSGL-1 engagement induces downstream signalling events that affect T-cell phenotype and function[44]. Deletion of PSGL-1 increased T-cell proliferation and exacerbated inflammation in vivo[48]. We also show that PMPs affect Treg proliferation, in particular within the CCR6$^+$HLA-DR$^+$ subpopulation that was preferentially targeted by PMPs. Thus, cell proliferation may be one of the mechanisms by which PMPs prevent IL-17 production by Tregs, which requires sufficient proliferation[9]. Previously, activated washed platelets were shown to attenuate T-cell proliferation[49,50]; in our coculture system, we show that TRAP-activated washed platelets can inhibit proinflammatory cytokine production by Tregs. This might be due, at least in part, to PMP formation during coculture. Other scenarios that might explain the inhibitory effect include the involvement of additional signalling pathways next to PSGL-1.

Our finding that CXCR3 signalling may be involved to a certain extent validates this line of thinking and deserves further attention, particularly because PF4, primarily expressed by platelets, triggers CXCR3-mediated signalling in activated T cells[31], leading to inhibition of proliferation and IFN-γ secretion[32], and was found to be a negative regulator of Th17 differentiation[33]. Other possibilities include the transfer of genetic information from PMPs to Tregs5 or the initiation of Treg–Treg signalling by PMPs binding to multiple Tregs[30]. The involvement of other adhesion molecules should also be considered, as exemplified by the ability of CD18 to keep Treg differentiation into IL-17–producing cells in check[51].

Most PMPs in the circulation do not originate from platelets but are produced by megakaryocytes in the bone marrow[52,53]. These megakaryocyte-derived microparticles are abundant in healthy individuals[3], whereas PMPs, identified by the expression of platelet activation markers P-selectin and/or CD63, are only observed during disease[29,42]. This implies that although megakaryocyte-derived microparticles have a more systemic function, PMPs may act locally at sites of platelet activation and that each has its own distinct role in homeostasis.

Apparently, different platelet activation stimuli can yield regulatory PMPs, as we observed that PMPs formed by TRAP-activated washed platelets were as able as stored platelet concentrate–derived PMPs to curb Treg differentiation. We started out by using platelet apheresis units as the main source for our PMPs, because they are known to generate relatively large quantities of PMPs in a controlled and sterile environment[24]. The majority of the isolated PMPs was P-selectin positive and thus originated from activated platelets in the concentrates. Therefore, our findings also add to the understanding of PMPs in platelet transfusion medicine, of which little is known thus far[54].

Platelets[55] and their microparticles[6] have been reported to exert a proinflammatory function in the autoimmune diseases lupus and rheumatoid arthritis, respectively. However, a recent study shows that platelets can exert either a pro- or anti-inflammatory effect, depending on the type of inflammatory response they encounter[2]. This observation, together with the alternative origin of the PMPs and a distinct cellular target (Tregs), could explain why we observe an anti-inflammatory effect instead. Of interest is our finding that binding of plasma-derived PMPs from HIV-infected individuals to Tregs was diminished in vitro. This suggests altered PMP binding, which although not directly obvious in peripheral blood, may be of relevance in the local tissue environment. Of note, increased IL-17 production by classical and nonclassical T cells is an increasingly recognized feature in HIV patients[56,57].

In conclusion, we have identified a novel function of PMPs to selectively bind a specialized subset of Th17-like progenitor cells in the Treg compartment and inhibit their differentiation into potentially pathogenic effector cells. The direct involvement of the cell adhesion molecule P-selectin and its ligand PSGL-1 in this process suggests a role for PMPs in wound healing by participating in the regulation of the required inflammatory response.

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Supplementary methods

Antibodies

The following conjugated mAbs were used for flow cytometry of Treg:

Surface - CD4(MT310)-FITC, CD27(M-T271)-FITC (DAKO), CD25(M-A251)-PE or APC, CCR6(11A9)-PE, CXCR3(1C6/CXR3)-PE-Cy5 (BD Biosciences), CXCR3(G025H7)-APC-Cy7, CD27(O323)-APC-Cy7, CD39(A1)-BV421, CD41(HIP8)-PerCP-Cy5.5 (BioLegend, San Diego, CA), PSGL-1(FLEG)-APC, CD25(BC96)-PE-Cy7, CD127(eBioRDR5)-PE-Cy7 (eBioscience, Uithoorn, The Netherland), CD4(SFCI12T4D11)-ECD, PE-Cy7 or PB, CD27(1A4CD27)-PE-Cy5.5, CD41(P2)-FITC or PE, CD62L(DREG56)-ECD, CD127(R34.34)-APC-AlexaFluor700, HLA-DR(Immu-357)-ECD (Beckman Coulter) and CD161(191B8)-APC (Miltenyi).

Intracellular - FOXP3(PCH101)-FITC or eFluor660, IL-17A(EBIO64DEC17)-AlexaFluor647 or APC-eFluor780, IFN-γ(4S.B3)-PE-Cy7, RORγt(AFKJS-9)-APC (eBioscience) and T-bet(4B10)-PE (Santa Cruz Biotechnology, Santa Cruz, CA).

PMP analysis using state-of-the-art flow cytometry

PMP purity/quantity was assessed using an optimized microparticle detection protocol (see Figure S1) on the GalliosTM flow cytometer (Beckman Coulter) adapted from a widely used method of Lacroix, Dignat-George and colleagues . Combined Annexin V-FLUOS (Roche, Basel, Switzerland) or Annexin V-AlexaFluor647 (Invitrogen) and anti-CD41(P2)-FITC, PE (Beckman Coulter) or anti-CD41(HIP8)-PerCP-Cy5.5 (BioLegend) mAb staining was used to identify PMPs. Additional mAbs used for PMP characterization were: P-selectin(AK-4)-FITC mAb (eBioscience), CD40(5C3)-PE (BD Biosciences) and CD63(H5C6)-PE-Cy7 (BioLegend). All staining solutions were centrifuged at 20.000g and 4°C for 20 min prior to use to remove fluorescent aggregates. Matched isotype controls were used to assess aspecific staining.

Size exclusion gating for PMPs was based on measurements using G100, G250, G500 Fluoro-Max Dyed Green Aqueous Fluorescent Particles (100, 250 and 500 nm; Duke Scientific Corp, Palo Alto, CA) and sulfate latex microspheres (900 nm; Invitrogen). Noise was excluded and washed Flow-Count Fluorospheres (~10 µm; Beckman Coulter) were added for PMP quantification (identified based on size and fluorescence).

PMP-associated P-selectin neutralization

P-selectin neutralization was performed by incubating PMPs for 30 min at room temperature in calcium-free Ringer's solution containing anti-P-selectin(AK-4)-FITC mAb (eBioscience), which is known to neutralize P-selectin (**Li et al, J.Surg.Res. 1996;61:543-548**). PMPs were pelleted by centrifugation and washed with calcium-free Ringer's solution.

Transwell assay and CD40 and CXCR3 neutralization

CD40 and CXCR3 neutralization on Treg was performed by the addition of an antagonistic anti-CD40 mAb kindly provided by Dr. M. de Boer (1 µg/mL; Tanox Pharma B.V., Amsterdam, The Netherlands) or an antagonistic anti-CXCR3(Ab89255) mAb (1 µg/mL; Abcam, Cambridge, UK) 1 hour before the addition of PMPs. ThinCerts™ cell culture inserts (0.4 µm; Greiner Bio-one, Wemmel, Belgium) for 24-well flat-bottom plates were used for the transwell assay, with the cells and the PMPs placed in the upper and the lower compartments, respectively.

Confocal laser scanning microscopy

Treg co-cultured with PMPs for 16 hours in the presence of anti-CD3/anti-CD28-coated-beads and recombinant human IL-2 were stained with CD41(P2)-FITC or anti-P-selectin(AK-4)-FITC and CD25(M-A251)-APC or PSGL-1(FLEG)-APC mAb antibodies, resuspended in HBS buffer pH 7.4 (10 mM HEPES, 135 mM NaCl, 5 mM KCl, 5 mM MgCl2, 1.8 mM CaCl2) and plated in 8-well microscopy chambers (Nunc, Wiesbaden, Germany). Images were acquired with a TCS SP5 confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with a HCX-PL-APO 63×1.2 water immersion lens while cells were maintained at 37°C. Leica LAS AF acquisition software was used.

FOXP3 gene methylation

Genomic DNA was isolated from Treg using the QIAamp DNABlood Mini kit (Qiagen, Hilden, Germany), treated by the EpiTect Bisulfite kit (Qiagen) and amplified using bisulfite-specific polymerase chain reaction (PCR) (forward 5' TGGATATTTGGTTAGAGTTAAGAAT 3' and reverse 5' ACCTAACACTCTCAAAACTTCAAAC 3'). The purified PCR product was labeled using BigDye Terminator version 1.1 Cycle Sequencing (Life Technologies, Carlsbad, Germany), purified using Sephadex G-50 FineDNA Grade (GE Healthcare, Little Chalfont, United Kingdom), sequenced on an ABI 3130 Genetic Analyzer (Life Technologies), and analyzed using Sequencing Analysis version 5.4 software (Life Technologies).

Washed platelet isolation and activation

Washed platelets (WP) were obtained from freshly collected whole blood of healthy donors anticoagulated with 3.2% sodium citrate (Becton Dickinson, Franklin Lakes, NJ) upon written informed consent. Whole blood was centrifuged for 15 min at 156g without brake to obtain platelet-rich-plasma (PRP). PRP was supplemented with 10% acid citrate dextrose (ACD) and centrifuged twice at 330g without brake for 20 min. The pellets were resuspended in 0.22 µm filtered HEPES tyrode (HT) buffer (NaCl 145mM, KCl 5mM, Na2HPO4 0.5mM, MgSO4 1mM, HEPES 10mM, Glucose 5mM, pH 7.3). Prostacyclin (10ng/ml; PGI2, Cayman Chemical, Ann Arbor, MI) was added after the first wash step to prevent platelet activation during centrifugation. After isolation washed platelets were rested for 60 min at 37°C. WP (100×109/L) were activated for 30 min with Thrombin Receptor Activator Peptide 6 (100µM;

TRAP; Sigma-Aldrich, Zwijndrecht, The Netherlands). After stimulation WP were diluted and resuspended in culture medium.

PMP generation by washed platelets

For PMP generation from freshly isolated WP, 2.5 mM calcium was added to WP (100×109/L) together with TRAP (100µM) in 0.22 µm filtered HT buffer and stimulated for 1 hour on a rolling device (shear stress) for optimal PMP generation. Subsequently, WP were diluted in 0.22µm filtered HT-buffer and PMPs were isolated as described earlier and washed using 0.22µm filtered calcium-free Ringer's solution. The TRAP generated PMPs were resuspended in filtered calcium-free Ringer's solution, snap-frozen with liquid N2 and stored at -80°C until use. Microparticle purity and quantity were assessed by flow cytometry as described earlier.

Suppression assay

The suppressor capacity of regulatory cells was studied in a co-culture suppression assay. FACS sorted CCR6+ Treg cultured with and without PMPs for 7 days (as described in Methods) were washed and rested overnight in fresh culture medium containing 25 U/mL recombinant human IL-2. Autologous CD4+CD25- T cells (2.5×104, from frozen) were stimulated with anti-CD3/anti-CD28-coated beads (Invitrogen, Breda, The Netherlands) in a bead:cell ratio of 1:5, in the absence or presence of increasing numbers of Tregs. T cell proliferation was analyzed by [3H]-thymidine incorporation using a Gas Scintillation Counter (Matrix-96 Beta-counter; Canberra-Packard, Meriden, CT). To this end, 0.037 MBq (1 Ci) [3H]-thymidine (ICN-Pharmaceuticals, Irvine, CA) was added to each well, cells were harvested after 8 hours of culture, and [3H]-thymidine incorporation was measured. The [3H]- thymidine incorporation is expressed as mean plus or minus SD counts per 5 minutes of at least triplicate measurements.

Peripheral blood staining

The following conjugated mAbs were used: CD4(RPA-T4)-APC-H7, CD25-BB515 (BD Biosciences), CD41(HIP8)-PE-Cy7, CD127(eBioRDR5)-PerCP-Cy5.5, FOXP3(PCH101)-eFluor660 (eBioscience) and P-selectin(AK-4)-PE (BioLegend). In brief, 200 µL blood was incubated with antibodies for 40 min. Intracellular staining for FOXP3 was performed after simultaneous red blood cells lysis and leukocyte fixation/permeabilization using 1 mL Fix/Perm reagent (eBioscience). Cells were then analyzed within 2 hours using the NaviosTM (Beckman Coulter) or LSRII (BD Biosciences) flow cytometers.

Plasma-derived microparticle isolation and co-culture with Treg

Plasma-derived microparticles were isolated from patient and healthy volunteer peripheral blood (EDTA) under sterile conditions using differential centrifugation. In brief, the peripheral blood was centrifuged at 1000g for 5 min. Plasma was then centrifuged at 1500g for 20 min to obtain platelet poor plasma (PPP). The PPP was divided in 1 mL aliquots and centrifuged for 20 min at 20.000g to pellet microparticles. The microparticle pellet was then resuspended in ~10 µL PPP, snap-frozen with liquid N2, and stored at -80°C. Upon thawing microparticles were washed with 0.22 µm filtered calcium-free Ringer's solution and analyzed as described for PMPs above.

For co-culture with Treg, microparticles were thawed and washed once with culture medium, before being cultured for 16h with Treg in culture medium containing recombinant human IL-2. PMP (CD41+P-selectin+) binding to Treg was assessed as described in Methods.

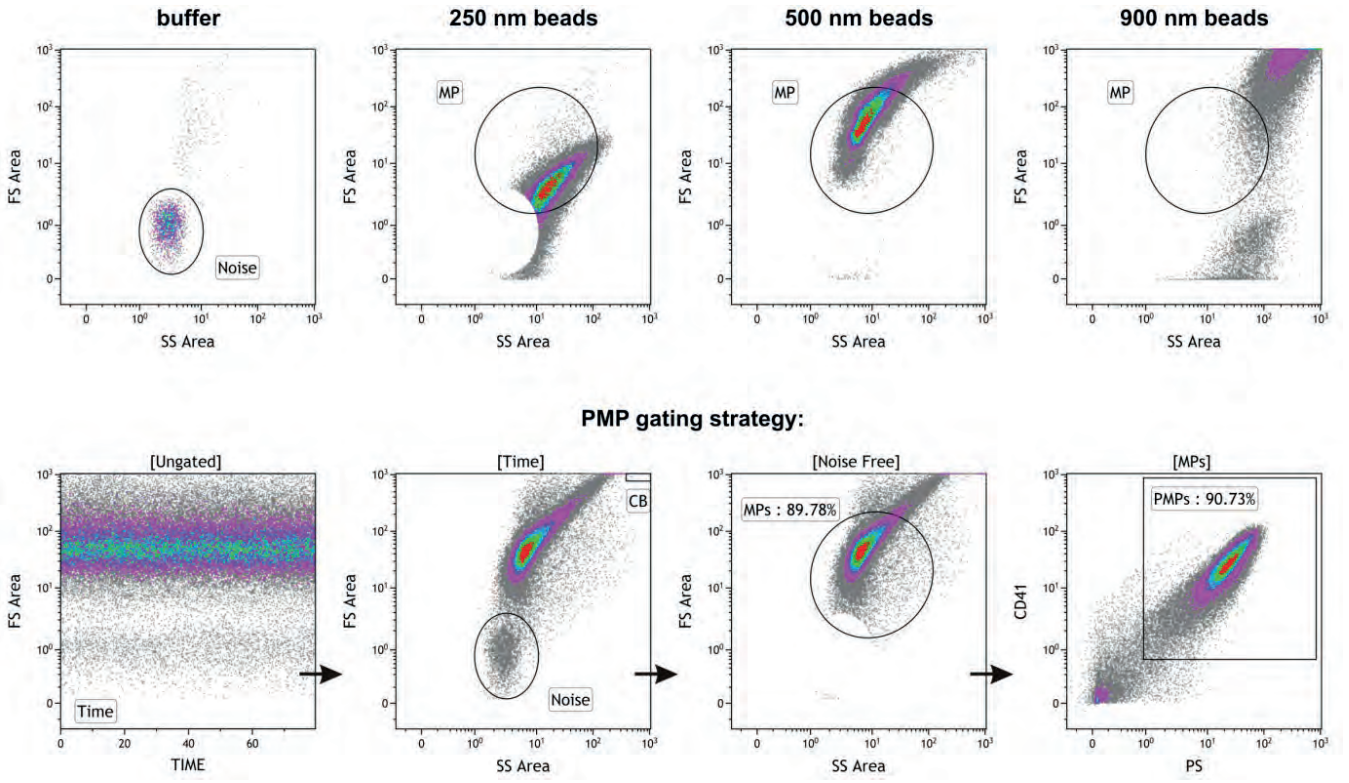

Figure S1. PMP measurement using the Gallios flow cytometer. PMPs ($CD41^+PS^+$) were purified from platelet apheresis units of healthy donors by differential centrifugation and analyzed on a Gallios flow cytometer for phosphatidylserine (PS) and CD41 exposure as described in Methods. Noise was excluded, size beads (250, 500 and 900 nm) were used to set a size exclusion gate, and fluorescent count beads ("CB", ~10 µm) were added for quantification (identified by size and fluorescence). Representative dot plots are shown.

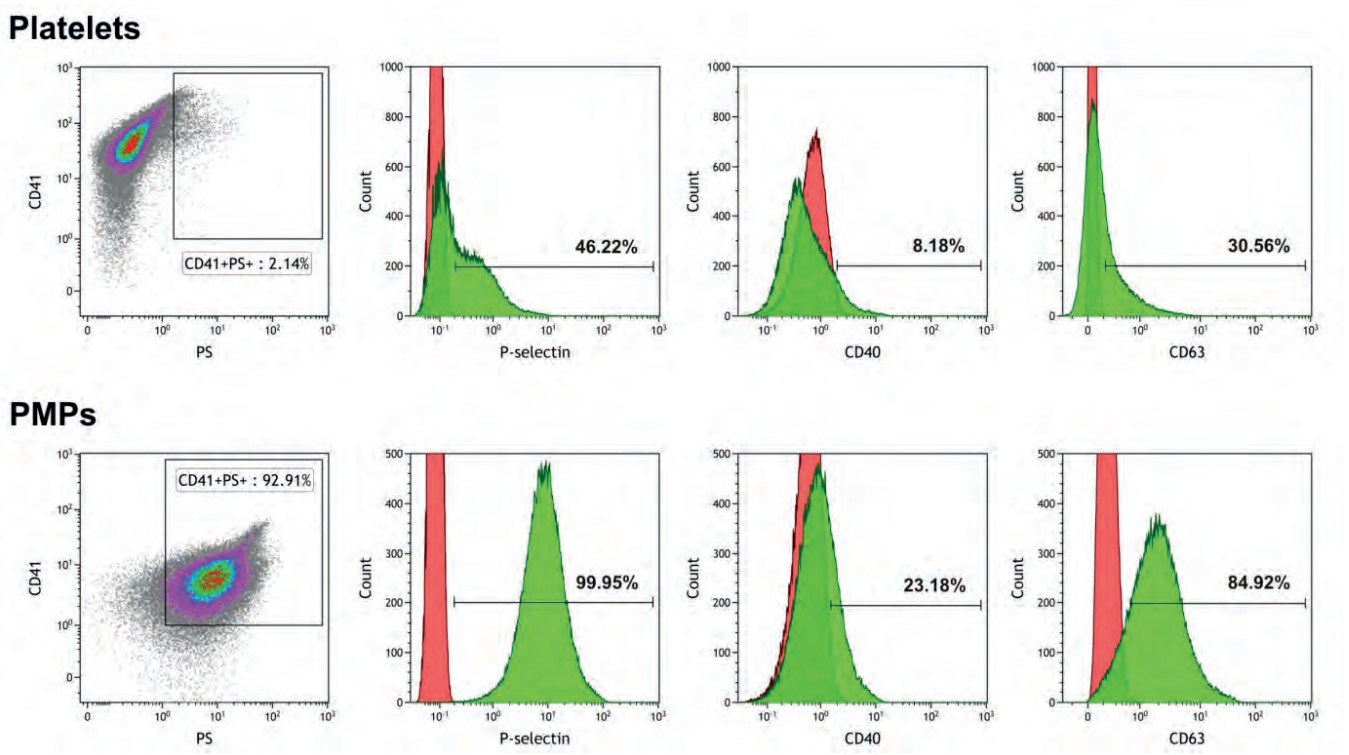

Figure S2. Phenotypic characterization of platelets and PMPs from a stored platelet concentrate. Platelets and PMPs were isolated from a 7-day-old platelet apheresis unit by differential centrifugation as described in the Methods. They were then characterized on a Gallios flow cytometer based on exposure of phosphatidylserine (PS), CD40, CD41, CD63 and P-selectin. Matched isotype controls are shown in red. The data is representative for three platelet concentrates.

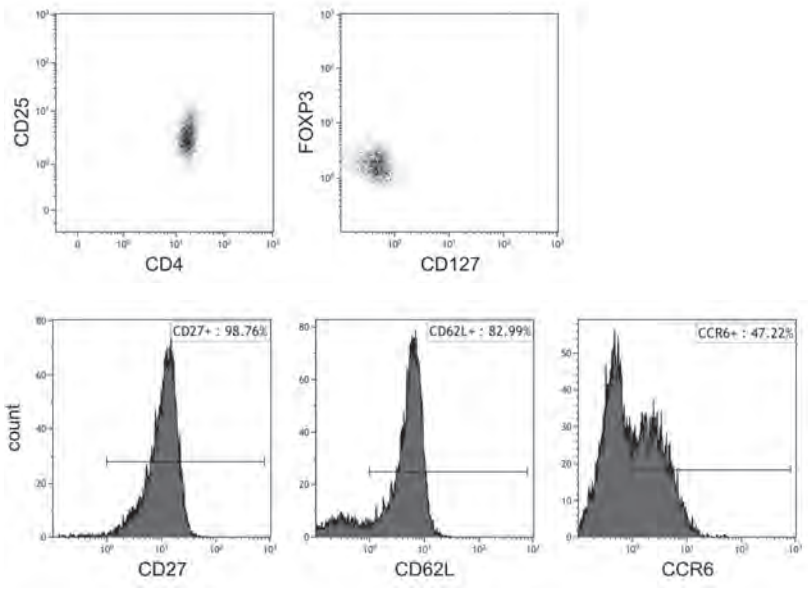

Figure S3. CD4⁺CD25^{high}CD27⁺CD127^{low}FOXP3⁺ Treg cell sorting. After CD4⁺ T cell isolation from PBMCs by magnetic bead sorting, CD4⁺CD25^{high} T cells were purified by fluorescence-activated cell sorting. The sorted cells were checked for surface CD4, CD25, CD27, CD62L, CD127, CCR6, and intracellular FOXP3 expression. A representative example of the flow cytometry analyses of these cells is shown.

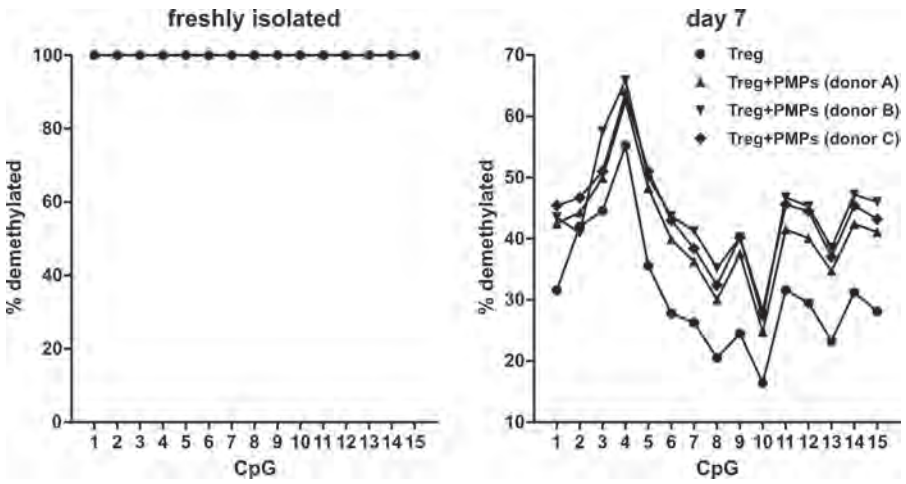

Figure S4. PMPs prohibit CpG methylation in the Treg cell-specific demethylation region of the *FOXP3* gene. *CpG methylation in the Treg cell-specific demethylation region of the FOXP3 gene in freshly isolated Treg (n=3 donors) and Treg that were cultured for 7 days with anti-CD3/anti-CD28-coated beads and recombinant human IL-2, IL-15, and IL-1β in the presence or absence of 1.5×10⁶ allogeneic PMPs isolated from platelet apheresis units of three healthy donors (n=3 PMP donors).*

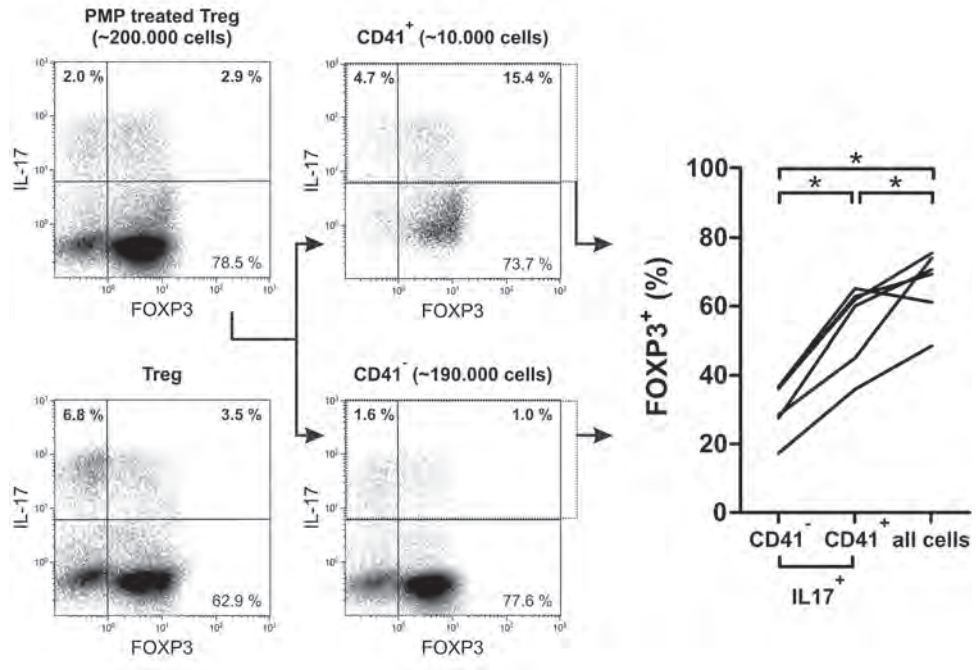

Figure S5. PMPs are enriched on IL-17⁺FOXP3⁺ compared to IL-17⁺FOXP3⁻cells. Treg were isolated from six healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads and recombinant human IL-2, IL-15, and IL-1β in the presence or absence of 1.5×10⁶ allogeneic PMPs isolated from platelet apheresis units of three healthy donors. Surface CD41 staining and intracellular FOXP3, IL-17 and IFN-γ staining was performed after stimulation with PMA and ionomycin, in the presence of Brefeldin A. Example IL-17/FOXP3 density plots of all cells and CD41⁻ and CD41⁺ Treg are shown. A density plot of Treg cultured without PMPs is shown as reference. Each donor's Tregs are plotted as a separate data series (line) representing the mean response of those Tregs to the three different donor's PMPs. *P<0.05

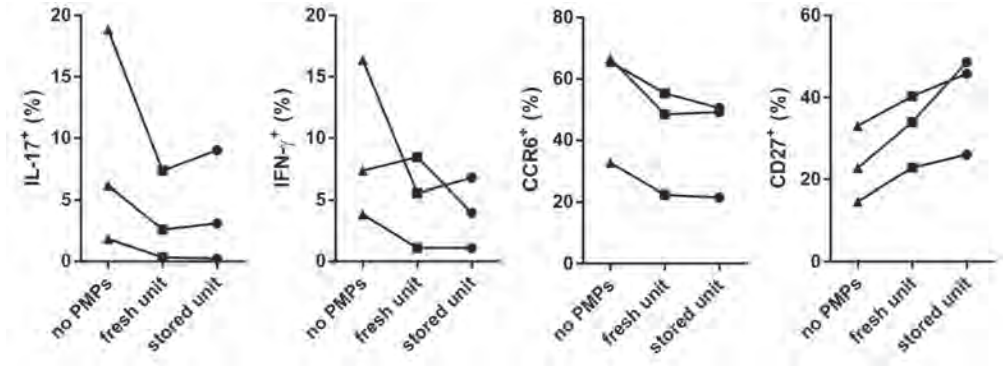

Figure S6. PMPs from fresh platelet apheresis units also inhibit IL-17 and IFN-γ production by Treg. Tregs were isolated from three healthy donors and cultured for 7 days with anti-CD3/anti-CD28-coated beads and recombinant human IL-2, IL-15 and IL-1β in the presence or absence of 1.5×10^6 allogeneic PMPs isolated from two-day (fresh) or seven-day stored platelet apheresis units from three healthy donors. Flow cytometry analyses of surface CCR6, CD27 and intracellular IL-17 and IFN-γ were performed after stimulation with PMA and ionomycin in the presence of Brefeldin A. Each donor's Tregs are plotted as a separate data series (line) with each data point representing the mean response of those Tregs to the three different donor's PMPs.

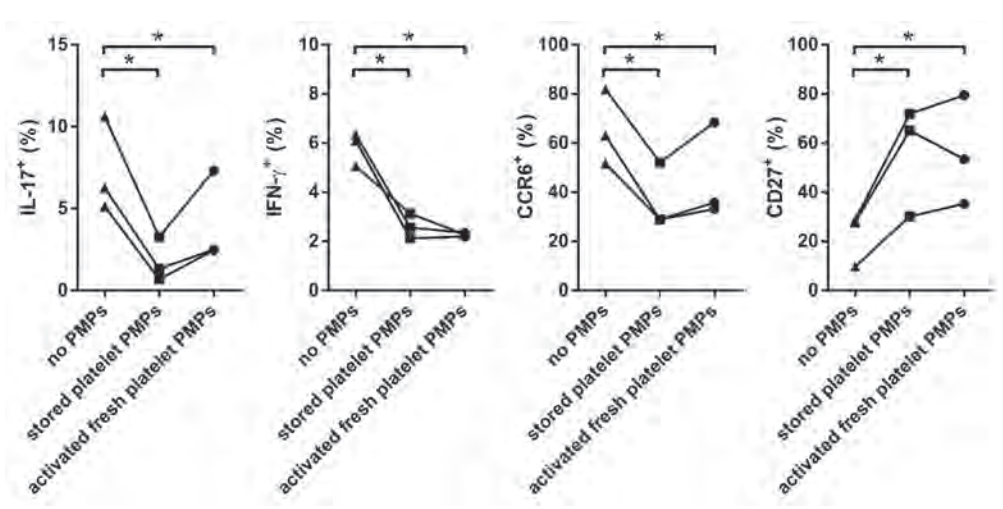

Figure S7. PMPs produced by TRAP-activated platelets also inhibit IL-17 production by Treg. Tregs were isolated from three healthy donors and cultured for 7 days with anti-CD3/anti-CD28-coated beads and recombinant human IL-2, IL-15 and IL-1β in the presence or absence of 1.5×10^6 allogeneic PMPs isolated from a stored platelet apheresis concentrate or from TRAP-activated washed platelets from three healthy donors. Flow cytometry analyses of surface CCR6, CD27 and intracellular IL-17 and IFN-γ were performed after stimulation with PMA and ionomycin in the presence of Brefeldin A. Each donor's Tregs are plotted as a separate data series (line) with each data point representing the mean response of those Tregs to the three different donor's PMPs. *P<0.05

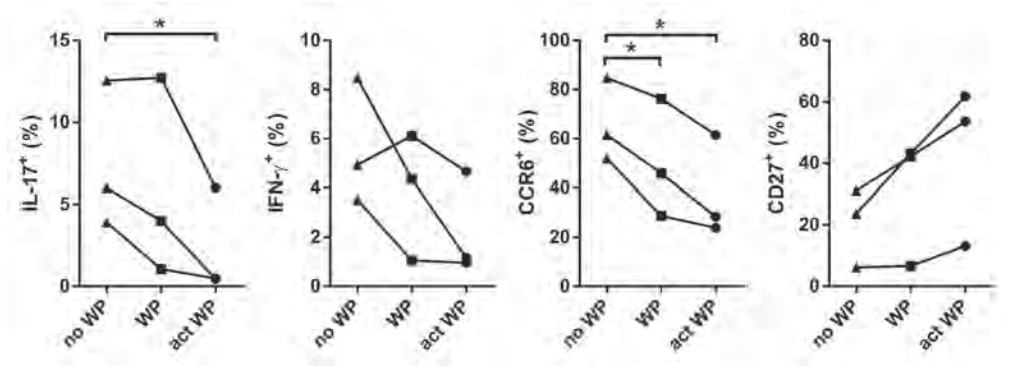

Figure S8. Activated washed platelets also inhibit IL-17 production by Treg. Tregs were isolated from three healthy donors and cultured for 7 days with anti-CD3/anti-CD28-coated beads and recombinant human IL-2, IL-15 and IL-1β in the presence or absence of 1.5×10^6 allogeneic resting (WP) or TRAP-activated washed platelets (act WP) from three healthy donors. Flow cytometry analyses of surface CCR6, CD27 and intracellular IL-17 and IFN-γ were performed after stimulation with PMA and ionomycin in the presence of Brefeldin A. Each donor's Tregs are plotted as a separate data series (line) with each data point representing the mean response of those Tregs to the three different donor's platelets. *P<0.05

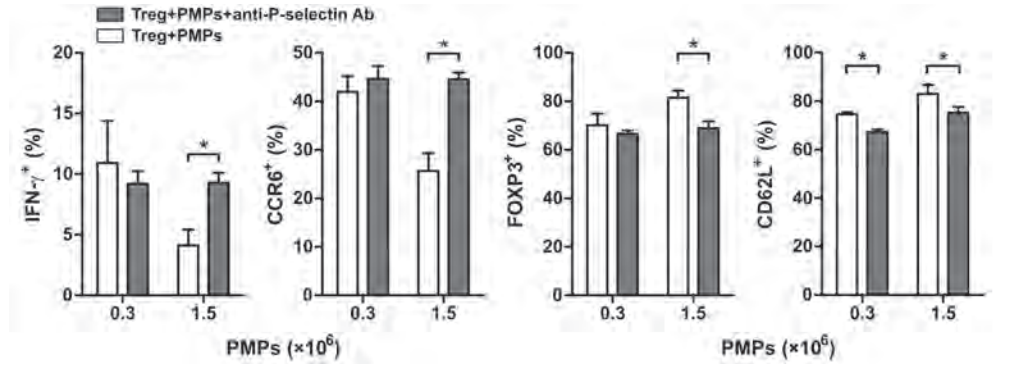

Figure S9. PMP inhibition of Treg differentiation is P-selectin-dependent. Treg were isolated from two healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads and recombinant human IL-2, IL-15, and IL-1β in the presence of allogeneic untreated or anti-P-selectin mAb-blocked PMPs isolated from platelet apheresis units of three healthy donors. Flow cytometry analyses of surface CCR6 and CD62L and intracellular FOXP3 and IFN-γ were performed after stimulation with PMA and ionomycin in the presence of Brefeldin A. Mean and SD from a representative Treg donor are shown. *P<0.05.

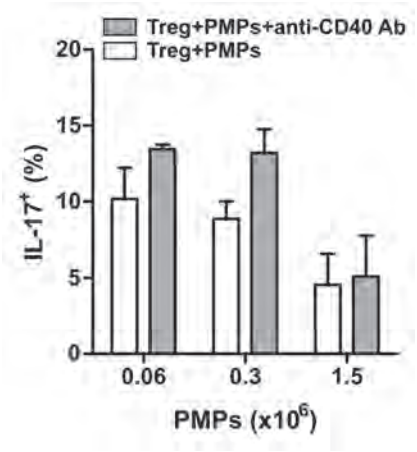

Figure S10. CD40 neutralization does not affect PMP inhibition of Treg differentiation. Treg were isolated from two healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads, recombinant human IL-2, IL-15, and IL-1β and allogeneic PMPs isolated from platelet apheresis units of three healthy donors in the presence or absence of anti-CD40 antagonistic mAb. Flow cytometry analysis of intracellular IL-17 was performed after stimulation with PMA and ionomycin in the presence of Brefeldin A. Mean and SD from a representative Treg donor are shown.

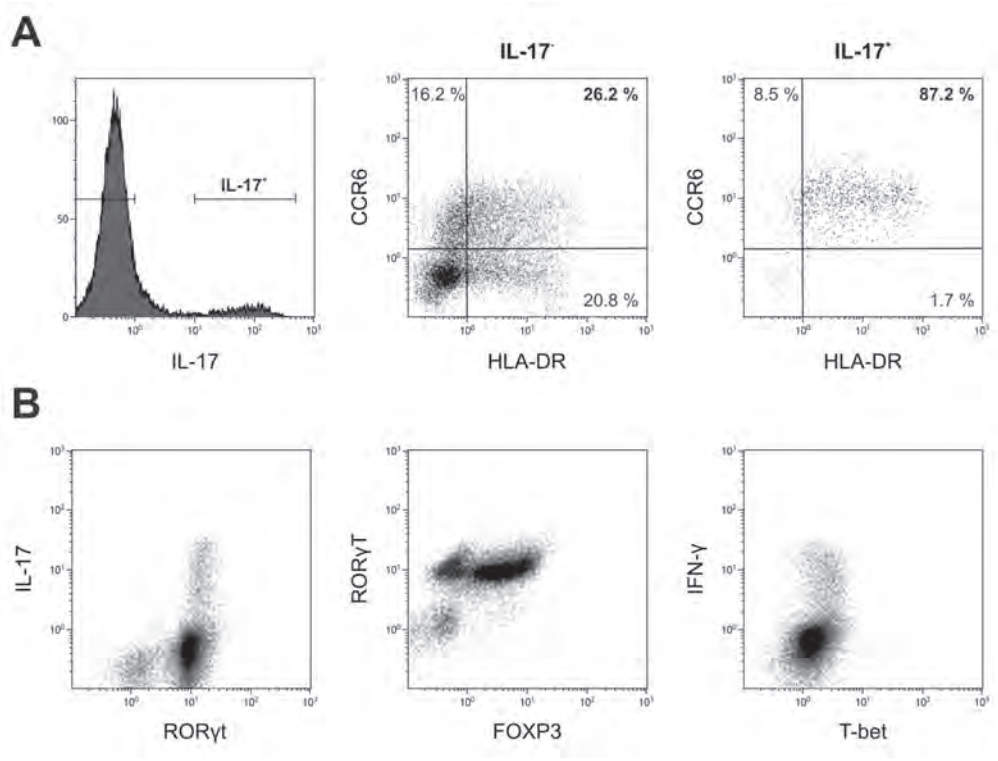

Figure S11. IL-17-producing cells co-express CCR6 and HLA-DR. Treg were isolated from five healthy donors and cultured for 7 days with anti-CD3/anti-CD28-mAb-coated beads and recombinant human IL-2, IL-15, and IL-1β. Flow cytometry analyses of surface CCR6 and HLA-DR and intracellular IL-17, IFN-γ, FOXP3, RORγt and T-bet were performed after stimulation with PMA and ionomycin in the presence of Brefeldin A. (**A**) Density plots showing the CCR6 and HLA-DR expression of IL-17-negative and IL-17-positive cells. (**B**) Density plots showing IL-17, RORγt, FOXP3, IFN-γ and T-bet expression. Representative results are shown.

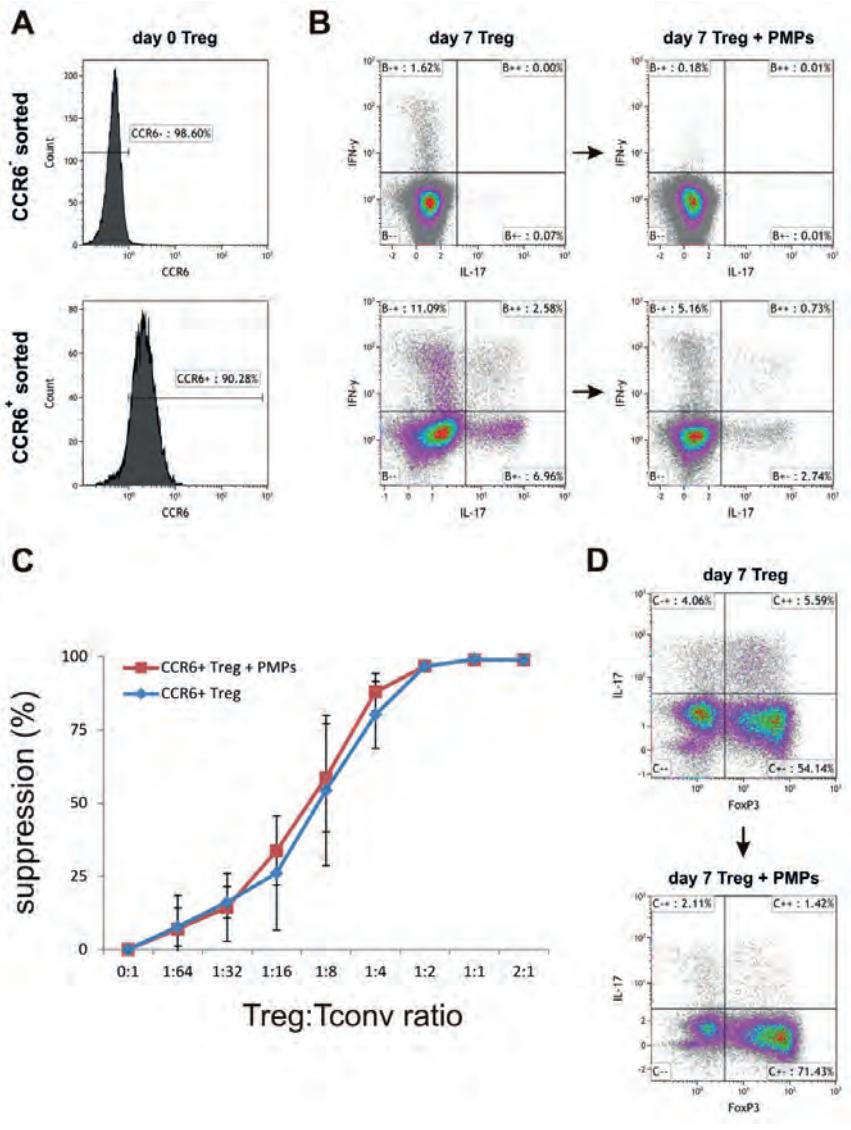

Figure S12. Suppression of T cell proliferation by CCR6⁺ Treg cultured with and without PMPs. CCR6⁺ and CCR6⁻ FACS sorted Treg from two healthy donors were cultured for 7 days in the presence of anti-CD3/anti-CD28-coated-beads and recombinant human IL-2, IL-15 and IL-1β in the presence or absence of 1.5×10^6 allogeneic PMPs isolated from platelet apheresis units of three healthy donors. (**A**) CCR6 expression on CCR6⁺ and CCR6⁻ sorted Treg on day 0. (**B**) Intracellular Treg expression of IL-17 and IFN-γ as assessed by flow cytometry on day 7. After 7 days the cultured CCR6⁺ Treg were washed and rested for a single day in fresh culture medium containing IL-2, after which a [³H]-thymidine-based Treg suppression assay was performed using autologous $CD4^+CD25^-$ responder cells as described in Methods. (**C**) Mean and SD of percent suppression of responder cell proliferation by CCR6⁺ Treg cultured with and without PMPs (n=3 donors) is shown. (**D**) Intracellular expression of IL-17 and FOXP3 by cultured CCR6⁺ Treg as assessed by flow cytometry on day 7 after stimulation with PMA and ionomycin, in the presence of Brefeldin A. Representative results from a single Treg donor are shown.

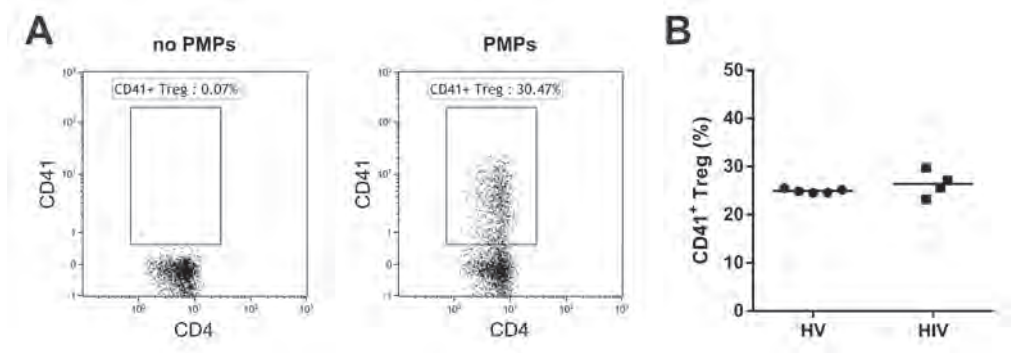

Figure S13. *Ex vivo* PMP binding to Treg is not affected by HIV-infection. $CD4^+$ T cells isolated from peripheral blood of HIV-infected adults on stable combination antiretroviral therapy (n=4) and of healthy volunteers (HV, n=5) were cultured for 16 hours with recombinant human IL-2 in the presence or absence of 1.5×10^6 allogeneic PMPs isolated from platelet apheresis units of three healthy donors. Flow cytometry analyses of CD4, CD127, CD25 and CD41 was performed. (**A**) Example of $CD41^+$ (PMP bound) $CD4^+CD25^{high}CD127^{low/-}$ Treg gating. (**B**) Percentage of $CD41^+$ Treg of HIV-infected adults and of healthy volunteers. Each patient's and healthy volunteer's Tregs are shown as a data point representing the mean response of those Tregs to the three different donor's PMPs.

Chapter 9

A randomized trial on the effect of anti-platelet therapy on the systemic inflammatory response in human endotoxemia

Dorien Kiers, Wouter van der Heijden, Lisa van Ede, Jelle Gerretsen, Quirijn de Mast, Andre van der Ven, Saloua el Messaoudi, Gerard Rongen, Marc Gomes, Matthijs Kox, Peter Pickkers, Niels Riksen

Thromb Haemost 2017;117:1798-1807

Abstract

The use of acetylsalicylic acid (ASA) is associated with improved outcome in patients with sepsis, and $P2Y_{12}$ inhibitors have been suggested to also have immunomodulatory effects. Therefore, we evaluated the effects of clinically relevant combinations of antiplatelet therapy on the immune response in experimental endotoxemia in humans *in vivo*. 40 healthy subjects were randomized to seven days of placebo, placebo with ASA, ticagrelor and ASA, or clopidogrel and ASA treatment. Systemic inflammation was elicited at day seven by intravenous administration of *Escherichia coli* endotoxin. ASA treatment profoundly augmented the plasma concentration of pro-inflammatory cytokines, but did not affect anti-inflammatory cytokines. Addition of either $P2Y_{12}$ antagonist to ASA did not affect any of the circulating cytokines, except for an attenuation of the ASA-induced increase in TNFα by ticagrelor. Systemic inflammation increased plasma adenosine, without differences between groups, and although $P2Y_{12}$ inhibition impaired platelet reactivity, there was no correlation with cytokine responses.

Introduction

Platelet aggregation inhibitors are the pharmacological cornerstone in the primary and secondary prevention of cardiovascular events. In addition, it is increasingly recognized that platelets have potent immunomodulatory properties. Platelets respond to inflammatory stimuli by release of chemokines[1], and expression of P-selectin and CD40 ligand, which are important regulators of platelet-leukocyte interactions[2]. The commonly used platelet aggregation inhibitors acetylsalicylic acid (ASA) and the $P2Y_{12}$ receptor inhibitors (such as ticagrelor and clopidogrel) potentially interfere with these immunological effects and may thereby influence the clinical outcome of patients that encounter a severe infection.

Several cohort studies have evaluated the association between acetylsalicylic acid treatment and outcome in patients with sepsis and pneumonia (summarized in Supplemental Table 1). Acetylsalicylic acid (ASA) treatment has been associated with a reduced mortality in patients with sepsis admitted to the Intensive Care Unit (ICU) in four large cohort studies[3-6]. Furthermore, in a smaller study, the combination of ASA with macrolide treatment was associated with reduced mortality in patients with pneumosepsis[7]. Only one study did not identify an association of platelet inhibitory therapy with mortality in ICU patients with sepsis, but this study also included patients on alternative platelet inhibitors than ASA[8]. ASA treatment in patients with community acquired pneumonia has also been associated with a reduced need for ICU admission[9] and mortality[10].

Clinical data on the immunomodulatory effects of $P2Y_{12}$ receptor inhibitors is sparse. Treatment with clopidogrel added to ASA is associated with attenuated inflammatory markers in patients following coronary interventions[11,12] and other studies have demonstrated that clopidogrel use is associated with increased incidence of pneumonia[13] and post-coronary artery bypass surgical site infections[14]. A post-hoc analysis of the Platelet inhibition and patient Outcomes (PLATO) trial suggested a reduced mortality after pulmonary infections and sepsis in patients treated with ticagrelor and ASA, compared to patients treated with clopidogrel and ASA[15], although a beneficial effect on sepsis-related mortality of ticagrelor was not confirmed in a more recent randomized trial[16].

The immunomodulatory effects of platelet inhibitors have been investigated in more detail in experimental models of inflammation. Seemingly counterintuitive, inhibition of cyclooxygenase (COX) augmented cytokine release. A three day treatment with 650 mg ASA in healthy volunteers augmented the *ex vivo* cytokine response of whole blood stimulated with lipopolysaccharide (LPS)[17], and treatment with COX inhibitors amplified the pro-inflammatory response after *in vivo* LPS administration in mice[18] and healthy volunteers[19,20].

In contrast, $P2Y_{12}$ inhibitors exert anti-inflammatory effects. A single dose of ticagrelor in healthy volunteers mitigated *ex vivo* cytokine production by LPS-stimulated leukocytes[21]. Similarly, treatment with ticagrelor or clopidogrel attenuated circulating levels of pro-inflammatory mediators tumor necrosis factor (TNF)α, interleukin (IL)-6, and chemokine ligand 2 during human endotoxemia[22]. Importantly, in contrast to clinical practice, the $P2Y_{12}$ inhibitors were not combined with ASA treatment in this study.

Taken together, epidemiological data indicate that treatment with ASA is associated with a beneficial outcome after sepsis. For P2Y12-inhibitors, data shows less consistent effects. To gain more insight into the immunomodulating properties of the various antiplatelet agents that are used for cardiovascular prevention, we aim to study the effect of low-dose ASA alone and in combination with the $P2Y_{12}$ inhibitors clopidogrel and ticagrelor in a well-validated human in vivo model of systemic inflammation.

Materials and methods

Study design, subjects and study procedures

This prospective randomized open-label, blinded endpoint study was performed according to the declaration of Helsinki, and registered by clinicaltrials.gov identifier NCT01978158. After approval of the local ethics committee (CMO Arnhem-Nijmegen, the Netherlands), forty healthy male volunteers aged 18-35 years, provided written informed consent and were included after screening. All subjects had normal physical examination, electrocardiography and routine laboratory values. The main exclusion criteria were medication use, recent febrile illness and increased bleeding risk.

Randomization was performed after inclusion by opening of a sealed opaque envelope, prepared by a research nurse not involved in the study. Subjects were randomized to one of four study arms, i.e. ticagrelor (AstraZeneca, London, UK) and ASA (Apotex, Leiden, The Netherlands) (TA-group, n=10), clopidogrel (Sandoz, Berleben, Germany) and ASA (CA-group, n=10), dummy capsules ('placebo', *Apotheek Radboudumc, Nijmegen, the Netherlands)* and ASA (PA-group, n=10), or placebo only (P-group, n=10). A detailed description of the study protocol is provided in the Supplemental Methods. Briefly, subjects were treated for seven days after a loading dose on day one. Loading dosages were 180, 300, and 160 mg, and maintenance dosing schedules were 90 mg b.i.d., 75 mg q.d and 80 mg q.d for ticagrelor, clopidogrel, and ASA, respectively. Drug adherence was monitored by pill counts. The seventh day of study treatment, systemic inflammation was elicited by administration of purified lipopolysaccharide (US Standard Reference Endotoxin, Escherichia Coli O:113, Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD, USA)). Endotoxin was administered as a bolus of 1 ng/kg, followed by continuous infusion at 1 ng/kg/h for three hours. Blood was obtained before study treatment, immediately before LPS administration, and every hour until eight hours afterwards with an extra blood withdrawal at 1.5h after initiation of LPS administration.

Cytokine measurement

Plasma concentration of the cytokines TNFα, IL-6, IL-8, IL-10 and IL-1 receptor antagonist (IL-1RA) and the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemoattractant protein-1 (MCP-1), also known as C-C motif chemokine ligand 2 (CCL2), were determined batchwise in Ethylenediaminetetraacetic-(EDTA) anticoagulated plasma by a multiplex immunoassay assay according to the manufacturer's instructions (Bio-Plex, BioRad, Hercules, USA and Milliplex, Millipore, Billerica, USA).

Plasma adenosine

Plasma adenosine levels were determined as previously described[23] (see Supplemental Methods).

Assessment of P2Y12 inhibition and platelet activation and responsiveness

Specific effectiveness of $P2Y_{12}$ inhibition was determined by the level of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) in citrated blood using an ELISA-based assay (CY-QUANT VASP/$P2Y_{12}$, BioCytex, Marseille, France) according to manufacturer's instructions. $P2Y_{12}$ inhibition is expressed as platelet reactivity index (PRI%), with a value <50% considered as adequate platelet inhibition. Platelet activation and responsiveness were assessed in whole blood (see Supplemental Methods). Briefly, platelet responsiveness was assessed by ex vivo stimulation with either adenosine diphosphate (ADP, 3.4 or 125 µM) or crossed-linked collagen-related-peptide (CRP-XL, 39 or 625 ng/mL) and platelet expression of P-selectin, a measure of degranulation, was measured using flow cytometry.

Platelet-leukocyte complexes

Platelet-leukocyte complex formation was determined using flow cytometry. Citrated whole blood was incubated with anti-CD61 (PC7; Beckman Coulter, Brea, CA, USA), anti-CD14 (ECD; DAKO, Heverlee, Belgium), anti-CD16 (FITC; Biolegend, San Diego, CA, USA) and anti-HLA-DR (PC5; Beckman Coulter, Brea, CA, USA) within 2 hours after blood collection. Optilyse B (Beckman Coulter, Brea, CA, USA) was added after 20 minutes for fixation followed by distilled water for erythrocyte lysis. Monocytes and neutrophils were selected based on granularity (side-scatter, SS) and size (forward-scatter, FS) as well as their expression of CD16, CD14 and HLA-DR (see *Supplemental Figure* 1 for gating strategy). The platelet-monocyte and platelet-neutrophil complex formation were quantified based on the % of cells expressing the platelet marker CD61 on respectively monocytes (CD14-positive) and neutrophils

(CD16high,CD14low, HLA-DR negative). It is known that the amount of PMC's measured depend on the time window between blood withdrawal and analysis. Due to variation in time from blood withdrawal to analysis between time points, data are only suitable to compare groups per time point, and not to evaluate changes in time within groups.

Plasma thromboxane

Plasma thromboxane B2 (the stable metabolites of thromboxane A2) was measured in citrated plasma in the placebo and placebo-ASA groups using an ELISA (*Thromboxane B2 EIA Kit, Oxford Biomedical Research, Oxford, USA*).

Leukocyte and platelet counts

Platelet and leukocyte counts and differentiation were performed in EDTA-anticoagulated blood using routine analysis methods (Sysmex XE-5000, The Netherlands).

Ex vivo monocyte stimulation

Monocytes were isolated from buffy coats obtained from healthy donors after written informed consent (Sanquin Blood Bank, Nijmegen, The Netherlands) as previously described[24] using both Ficoll-Paque and hyper-osmotic Percoll density gradient centrifugation and washing with warm PBS for purity and low platelet contamination. Washed platelets were isolated as previously described[21],and pre-incubated with ASA (5 uM, Sigma) for 1 hour and washed to remove ASA. Supernatant and platelets were collected for co-incubation with isolated and adhered monocytes. Adhered monocytes (1.10^5) were incubated for 24 hours with Escherichia coli LPS (10ng/mL; serotype 055:B5, Sigma), prostaglandin E2 (0.1 uM or 1uM; Sigma), ASA (5-500uM), unexposed or ASA-exposed platelet supernatant (1:4 dilution) and/or unexposed or ASA-exposed washed platelets (ratio 1:50 or 1:25). Hereafter, supernatants were collected and stored at -20 degrees. ELISA was used to determine TNFα.

Statistical analysis

Data are expressed as a mean ± SEM or as median [interquartile range], according to their distribution. Data were tested for normality using the Shapiro-Wilk test. To limit the number of statistical tests used, three hypotheses were hierarchically defined and sequentially tested by performing repeated measures two-way ANOVAs (interaction term of group and time) on specific pairs of groups: 1. To evaluate the effects of ASA, placebo-ASA was compared to placebo 2. To evaluate the effect of clopidogrel or ticagrelor added to ASA, clopidogrel-ASA and ticagrelor-ASA were compared to placebo-ASA. 3. In case of an effect of ticagrelor or clopidogrel added to ASA, a comparison of clopidogrel-ASA with ticagrelor-ASA was made to assess for differences between these $P2Y_{12}$ inhibitors. In case no differences between groups were identified, no further statistical testing was performed. Statistical tests used are described in the figure legends and Supplement. All statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, USA). A two-sided p value <0.05 was considered statistically significant.

Results

Subject inclusion and follow up, drug safety, compliance, and subject characteristics

The forty subjects that were included all completed the study (see CONSORT [Consolidated Standards of Reporting Trials] flow chart in *Supplemental Figure 2*). Study treatment was well tolerated. One subject treated with clopidogrel-ASA reported a spontaneous haematoma and one subject treated with ticagrelor-ASA experienced three days of mild dyspnea with preserved exercise tolerance (both Grade I). Treatment compliance was 100%. Demographic characteristics are listed in Table 1.

Table 1. Demographic characteristics.

	Placebo	Placebo and acetylsalicylic acid	Ticagrelor and acetylsalicylic acid	Clopidogrel and acetylsalicylic acid
Age [y]	22 [20-28]	21 [19-24]	22 [21-24]	21 [20-21]
Height [cm]	183 [175-187]	186 [183-190]	180 [177-186]	186 [181-191]
Weight [kg]	77 [66-84]	83 [80-88]	72 [68-77]	83 [77-91]
BMI [kg/m²]	23 [21-25]	24 [23-26]	22 [21-24]	24 [22-25]

Data is presented as median [interquartile range]. y: years, cm: centimeter, kg: kilogram, BMI: body mass index, m: meter.

Cytokine and chemokine response

Plasma cytokine and chemokine concentrations were below the lower detection limit of the assay before administration of LPS. LPS administration resulted in an increased plasma concentration of the cytokines Tumor Necrosis Factor (TNF)α, Interleukin(IL)-6, IL-8, IL-10, IL-1 receptor antagonist (IL-1RA), monocyte chemoattractant protein(MCP)-1, macrophage inflammatory protein(MIP)-1α and MIP-1β in all subjects (Figure 1). To evaluate the effects of low dose acetylsalicylic acid, we compared the cytokine response of subjects treated with ASA to the placebo treated group.

Plasma levels of TNFα,IL-6, IL-8 and MIP-1α were increased to a greater extent in ASA treated subjects compared with the placebo group, and there was a trend towards enhanced levels of MCP-1. To evaluate whether addition of ticagrelor or clopidogrel further affected the systemic inflammatory response, we compared the cytokine response of the ASA group with the ticagrelor-ASA group and the clopidogrel-ASA group. Only for TNFα, addition of ticagrelor

to ASA mitigated the ASA-induced augmentation, addition of clopidogrel to ASA did not alter any of the ASA-mediated changes in cytokines. Comparison of the ASA-clopidogrel and ASA-ticagrelor group did not reveal any difference in TNFα levels between ticagrelor- or clopidogrel-treated subjects. None of the study treatments affected plasma concentrations of MIP-1β and the anti-inflammatory cytokines IL-10 and IL-1RA.

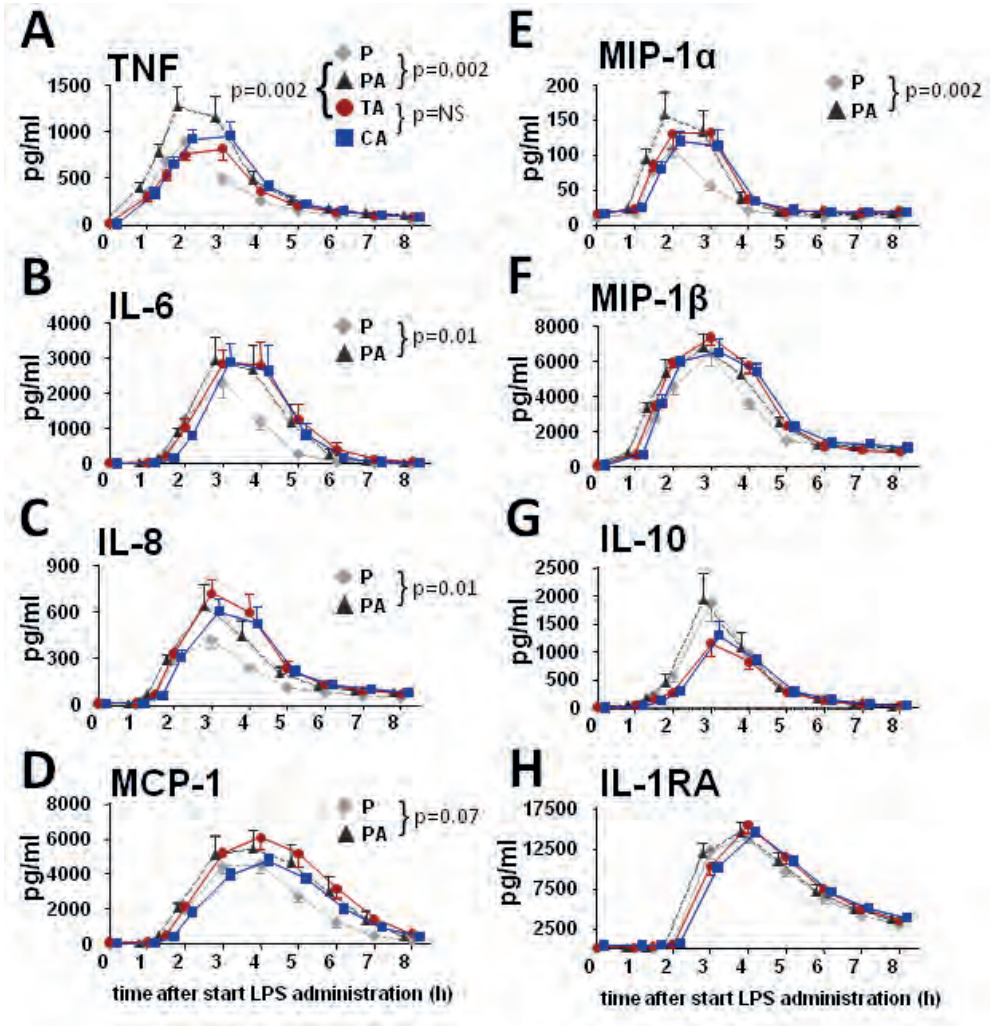

Figure 1. Plasma cytokine concentrations. Plasma concentrations of (**A**) TNFα, (**B**) IL-6, (**C**) IL-8, (**D**) IL-10 and (**E**) IL-1RA during endotoxemia. Data are expressed as means with SEM. Effects of study treatment on the cytokine response were evaluated using a two-way ANOVAs (interaction term of group and time) on group pairs. P-values of significant differences are displayed in the figure. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.

Plasma adenosine

As adenosine is known to have potent anti-inflammatory effects[25], and ticagrelor has previously been shown to increase extracellular adenosine by blocking reuptake[26], we evaluated the effect of the study treatments on plasma adenosine. Plasma adenosine levels were comparable at baseline between groups. Endotoxemia significantly increased adenosine levels to a similar degree in all groups (Figure 2), and there were no differences between groups.

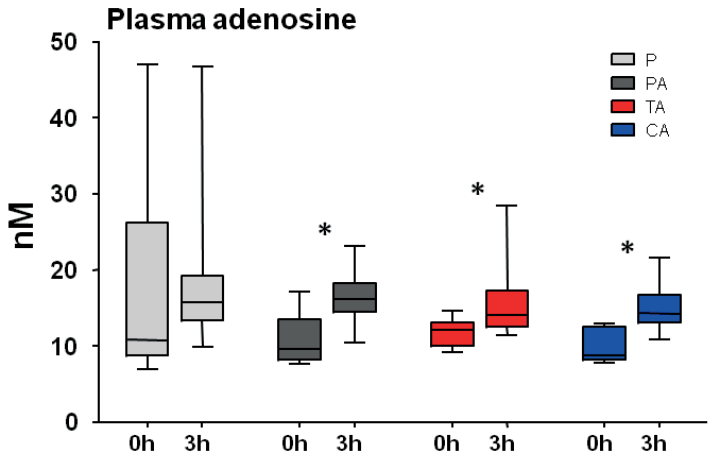

Figure 2. Plasma adenosine. Plasma concentrations of adenosine before and 3h after initiation of endotoxin administration. Data are expressed as box and whiskers plots with median, minimal and maximal observed values. Baseline values were comparable between groups (Kruskal-Wallis test, p=0.13), and changes in adenosine after 3h were tested with the Wilcoxon signed rank test. * indicates p<0.05.

Platelet $P2Y_{12}$ receptor inhibition and platelet activation and responsiveness

To evaluate the specific inhibition of $P2Y_{12}$, the level of phosphorylation of vasodilator-stimulated phosphoprotein (VASP) was measured. There was no $P2Y_{12}$ inhibition before the start of study treatment in any subject, and $P2Y_{12}$ dependent platelet inhibition remained unaffected throughout the study in the placebo and placebo-ASA groups (Figure 3). Ticagrelor treatment resulted in a VASP-PRI% <50% in all subjects, whereas platelet inhibition varied largely in clopidogrel-treated subjects.

In vivo platelet activation, as measured by unstimulated platelet P-selectin expression, was low in all subjects and was not affected by study treatment or endotoxemia (Figure 4A). Platelet responsiveness to low and high concentrations of adenosine diphosphate and cross-linked collagen-related peptide remained unaffected by both experimental

endotoxemia and ASA treatment (Figure 4B-E). Treatment with $P2Y_{12}$ inhibitors diminished adenosine diphosphate-induced P-selectin expression in clopidogrel- and ticagrelor-treated subjects similarly. P-selectin expression after stimulation with low concentration cross-linked collagen-related peptide was reduced in clopidogrel- and ticagrelor-treated subjects similarly. However, stimulation with high concentration cross-linked collagen-related peptide fully surmounted the clopidogrel-, but not the ticagrelor-induced reduction in platelet responsiveness. Platelet responsiveness did not correlate with cytokine responses (*Supplemental Figure 3*).

Platelet-leukocyte complexes

To evaluate whether antiplatelet agents affect platelet-monocyte and platelet-neutrophil interactions, the percentage of platelet-monocyte and platelet neutrophil complexes (PMC and PNC) was determined. Due to the endotoxin-induced transient monocytopenia, platelet-monocyte complexes were not measurable at 2 hours after onset of endotoxemia. Although there is a trend towards decreased PMC formation in the ticagrelor-ASA treated group at 4h, there were neither differences in formation of PMCs, nor of PNCs at any time point between groups (Figure 5). Again, there was no correlation between PMC and PNC formation and cytokine response (*Supplemental Figure 4*).

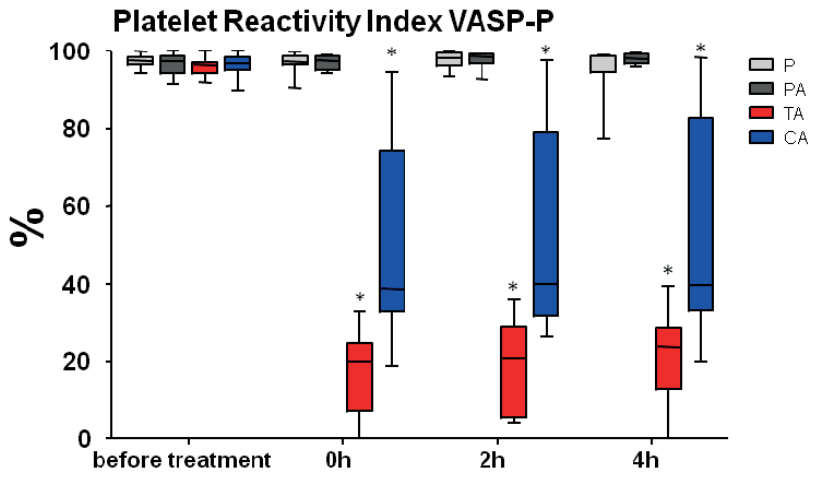

Figure 3. VASP-P Platelet Reactivity Index. Platelet Reactivity Index (PRI), expressed as the percentage of phosphorylation of vasodilator-stimulated phosphoprotein (VASP-P). Data are expressed as box and whiskers plots with median, minimal and maximal observed values. Baseline values were comparable among groups (Kruskal-Wallis test, p=0.53). Treatment with PA and P did not alter PRI (Friedman test, p=0.20 and p=0.16 respectively), treatment with TA and CA decreased PRI (Friedman test with post-hoc Dunn's Multiple Comparison Test p=0.0004 and p=0.006). * indicates a difference with baseline value.

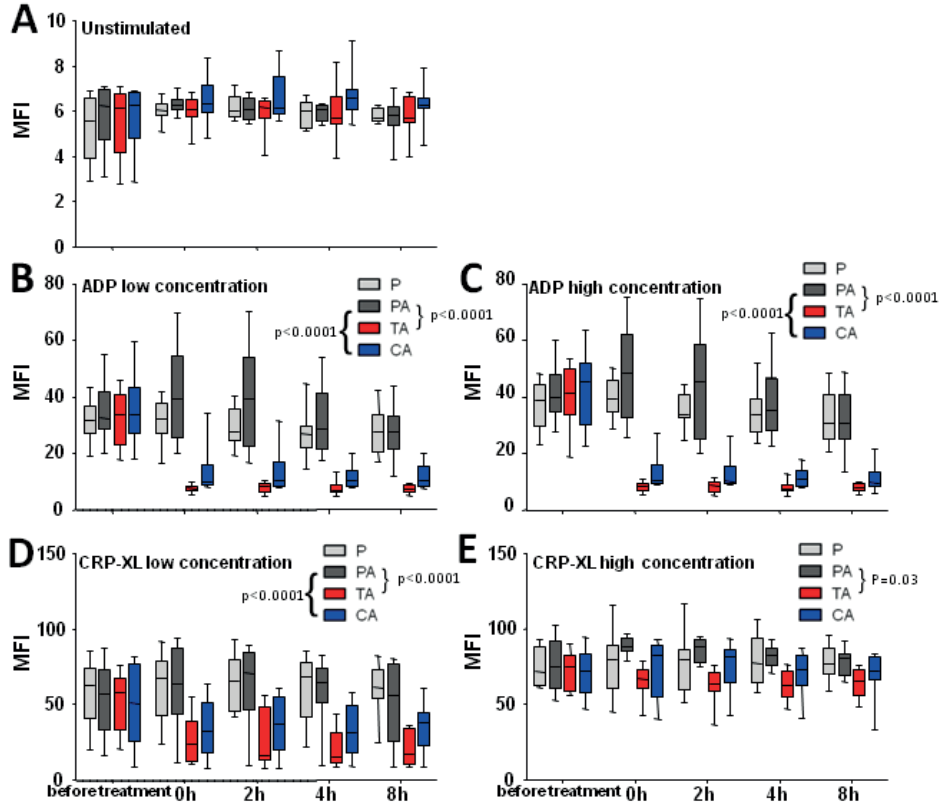

Figure 4. Platelet activation and responsiveness. Mean fluorescence intensity as a measure of platelet P-selectin expression before study treatment and during endotoxemia of unstimulated platelets (**A**), and after stimulation with low (**B**) and high (**C**) concentrations of adenosine diphosphate (ADP, 3.4 or 125 µM) and low (**D**) and high (**E**) concentrations of cross-linked collagen-related-peptide (CRP-XL, 39 or 625 ng/mL). Data are expressed as box and whiskers plots with median, minimal and maximal observed values. Effects of study treatment on platelet activation status and reactivation were evaluated using a two-way ANOVAs (interaction term of group and time) on group pairs. P-values of significant differences between groups are displayed in the figure. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.

Prostaglandins

To evaluate if COX-1 inhibition by ASA treatment was sufficient, plasma levels of thromboxane B2, the stable metabolite of the COX-1 product thromboxane A2, were determined. ASA treatment strongly reduced thromboxane B2 levels compared to placebo treatment (Figure 6).

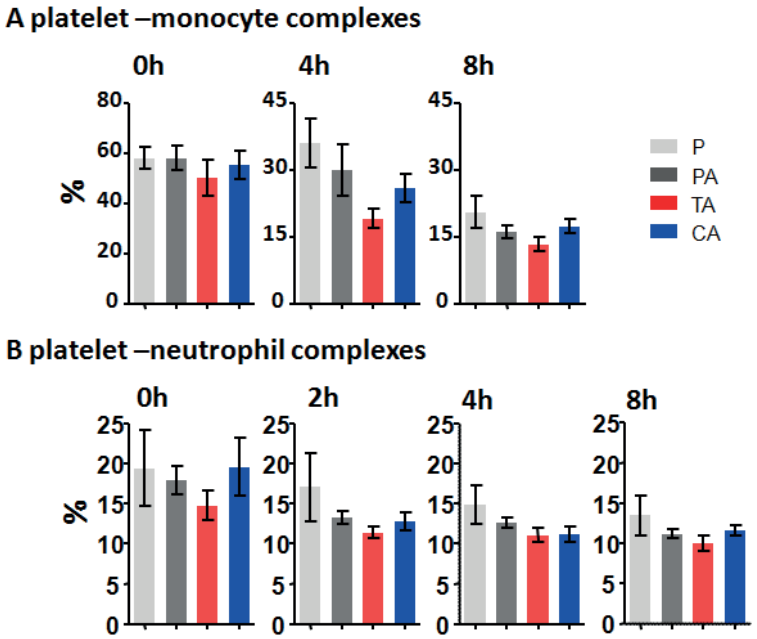

Figure 5. Platelet-leukocyte complexes. Percentage of platelet-monocyte complexes (**A**) and platelet-neutrophil complexes (**B**) during endotoxemia. Data are expressed as mean with SEM. Differences between study treatment groups were evaluated using a one-way ANOVA, there were no significant differences between groups. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.

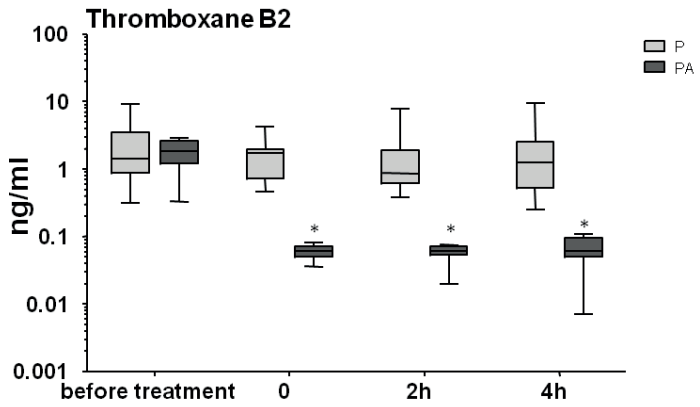

Figure 6. Thromboxane B. Plasma concentration of thromboxane B before and after treatment with placebo (P) or placebo with acetylsalicylic acid (PA). Data are expressed as box and whiskers plots with median, minimal and maximal values. Baseline values were comparable among groups (Mann-Whitney test, p=0.80).Treatment with ASA mitigated plasma thromboxane B (Friedman test with post-hoc Dunn's Multiple Comparison Test p=0.004). Endotoxemia did not affect thromboxane B levels (Friedman test p=0.83 and p=0.83 in P and PA groups, respectively). * indicates a difference with baseline.

Hemodynamic parameters, temperature and symptoms

Endotoxemia resulted in a decrease of mean arterial blood pressure of approximately 15 mmHg (*Supplemental Figure 5A*) and an increased heart rate with approximately 35 bpm (*Supplemental Figure5B*). Body temperature increased with 2.5°C (*Supplemental Figure 5C*). Endotoxin-related symptoms peaked at 3 hours and resided six hours after the start of endotoxin infusion (*Supplemental Figure 5D*). There were no differences in these parameters between groups.

Platelet and leukocyte count and differentiation

Platelet counts were comparable after study treatment (*Supplemental Figure 6A*) and endotoxemia resulted in a typical neutrophilia, and a transient thrombo-, mono- and lymphocytopenia (*Supplemental Figure 6B-D*), which were similar between groups.

Ex vivo monocyte stimulation

In order to evaluate whether the ASA-induced augmentation of the inflammatory response is platelet-dependent, isolated, adhered monocytes were stimulated with LPS in different conditions. Incremental concentrations of ASA did not affect TNFα production upon LPS stimulation, whereas PGE2 attenuated TNFα production (Figure 7A).Therefore, we hypothesized that platelets mediated the ASA-induced pro-inflammatory response. Correspondingly, platelets pre-exposed to ASA augmented LPS-stimulated monocytic TNFα production, (Figure 7B), and similarly the supernatant of ASA-exposed platelets augments monocytic TNFα response (Figure 7C). These effects were abrogated in the presence of PGE2.

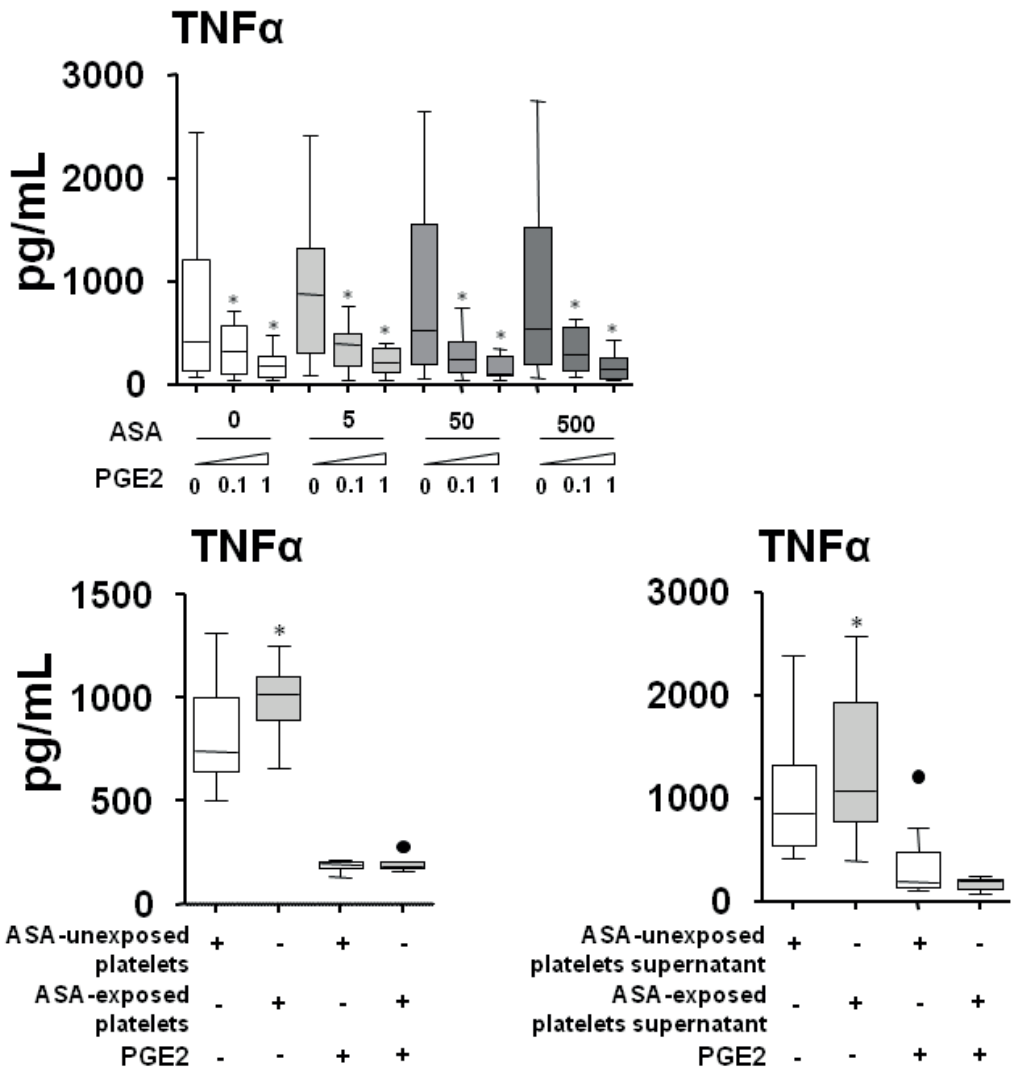

Figure 7. Ex vivo experiments with isolated monocytes, acetylsalicylic acid, prostaglandin E2 and platelets. TNFα production of LPS-stimulated monocytes with incremental dosages of ASA and/or PGE2 (**A**). Treatment with ASA did not affect TNFα production (Wilcoxon signed rank test, p=NS), whereas PGE2 attenuated TNFα production compared to the condition without PGE2 (Wilcoxon signed rank test, * indicates p<0.05, n=9). TNFα production of LPS-stimulated monocytes in the presence of unexposed or ASA-exposed platelets (n=9) (**B**) or the supernatant of these platelets (n=12) and PGE2 (1 uM) (**C**). Treatment of platelets with ASA augmented TNFα production (Wilcoxon signed rank test, * indicates p<0.05), PGE2 abrogated this ASA-platelet mediated effect. Data are expressed as box and whisker plots with median, minimal and maximal values. TNFα; Tumor Necrosis Factor-α

Discussion

We evaluated the effects of clinically relevant dosages of ASA, ticagrelor, and clopidogrel on systemic inflammation during human endotoxemia. A seven day course of low-dose ASA profoundly enhanced the pro-inflammatory response. Although the addition of ticagrelor attenuated the ASA-induced increase in TNFα levels, it did not affect the ASA-induced augmentation of other pro-inflammatory cytokines and chemokines. Furthermore, clopidogrel added to ASA did not affect the ASA-enhanced pro-inflammatory response during endotoxemia. There were no significant differences in immunomodulating effects between ticagrelor and clopidogrel added to ASA.

The pro-inflammatory effects of low-dose ASA observed in this study mirror results from previous experimental studies on COX-inhibitors in humans. Single or multiple high dose(650 mg) treatment with a ASA augmented LPS-induced pro-inflammatory cytokine production ex vivo[17,27] and treatment with the COX-inhibitor ibuprofen showed similar results during human endotoxemia[19,20]. Accordingly, ASA was shown to enhance plasma TNFα upon Salmonella typhi vaccination in healthy volunteers[28]. Interestingly, the timing of ASA treatment appears to be of importance, as a single dose of 1000 mg of ASA just before LPS administration did not affect the inflammatory response[18]. ASA, as a COX-inhibitor, abrogates the production of PGE2, and we and others have shown that PGE2 attenuates cytokine production[29]. Furthermore, the addition of exogenous PGE2 dose-dependently prevents the pro-inflammatory of ASA[30]. As such, ASA prevents PGE2 production and thereby abrogate it's inhibitory effect on cytokine production. Of note, low dose ASA and ibuprofen treatment both have shown to attenuate the production of PGE2 during human endotoxemia in vivo[31]. Of note, the studies that assessed the effect of COX-inhibition on LPS-induced cytokine production were performed in whole blood or in vivo, and therefore in the presence of platelets[17,27].

To investigate whether the ASA-induced anti-inflammatory effect depends on a direct effect on myeloid cells or is platelet dependent, we performed ex vivo experiments on human isolated monocytes and platelets. The data shows that that the ASA induced pro-inflammatory effect is mediated by platelets. This is in correspondence with previous evidence obtained from experiments with murine bone marrow derived macrophages (BMDM), in which it was demonstrated that TNFα production of BMDM was shown to be attenuated in the presence of platelets or platelet supernatant[32]. Analogous to our experiments , pretreatment with ASA of platelets abolishes the anti-inflammatory effects. These authors further demonstrate that platelets exert anti-inflammatory effects through the activation of the COX-1-PGE2 pathway[32].

Taken together, it is conceivable that the mechanism of the ASA-induced pro-inflammatory effects observed are caused by inhibition of PGE2 production by platelets. As a consequence, PGE2-induced dampening of pro-inflammatory cytokine responses is attenuated, ultimately resulting in increased cytokine levels.

Although the addition of ticagrelor to ASA significantly affected plasma TNFα levels during endotoxemia, it did not influence other cytokines or chemokines, and clopidogrel added to ASA did not affect the cytokine response at all. Furthermore, there were no statistical significant differences in cytokine responses between ticagrelor- and clopidogrel-treated subjects. Animal studies evaluating the role of $P2Y_{12}$ inhibition in various models of inflammation are sparse and have yielded conflicting results, showing pro-inflammatory[33], anti-inflammatory[34,35], or no effects[36]. Our results are in contrast with the scarcely available human data, as a single dose of ticagrelor reduced platelet-leukocyte interaction and *ex vivo* cytokine responses[21], and ticagrelor and clopidogrel reduced pro-inflammatory cytokine levels during human endotoxemia[22]. Importantly, subjects did not receive concomitant treatment with ASA in these studies. The anti-inflammatory effects observed in these studies were suggested to be caused by a reduction in platelet reactivity and reduced platelet-leukocyte interaction[22]. However, in our study, there were no differences between groups in platelet-leukocyte interactions. In addition, although ADP-dependent platelet responsiveness was reduced by $P2Y_{12}$ inhibitors, the degree of responsiveness did not correlate with cytokine responses. Therefore, it is most likely that the pro-inflammatory potential of ASA overshadows the previously observed immunomodulating effects of $P2Y_{12}$ inhibitors. This is important, as most patients use a $P2Y_{12}$ inhibitor in combination with ASA, and not as monotherapy. Various recent studies showed that ticagrelor increases the extracellular levels of endogenous adenosine[37,38]. Since adenosine is a potent anti-inflammatory agent, it was proposed that this mechanism contributes to the beneficial effect of ticagrelor on endotoxemia, but adenosine levels were not measured in this study[22]. We found no effects of ticagrelor on plasma adenosine, which is consistent with our previous finding that ticagrelor does not inhibit ENT transport at clinically relevant dosages[39]. In conclusion, our findings indicate that ticagrelor and clopidogrel do not relevantly modulate the immune response when they are combined with ASA treatment. This implies that that the beneficial effects of ticagrelor and ASA compared with clopidogrel and ASA on mortality after sepsis and pneumonia in the PLATO trial are unlikely caused by immunomodulation[15].

The putative immunomodulating effects of antiplatelet therapy are important for two reasons. First, use of anti-platelet therapy in the ICU population is very common, with estimates ranging from 17-43%[6,40-42]. ASA treatment has consistently been associated with reduced mortality in patients with sepsis in several studies[3-7], with the exception of one smaller study, which evaluated not only treatment with ASA, but also other anti-platelet therapy[8]. In patients with community acquired pneumonia, pretreatment with ASA has been associated with a reduced need for ICU admission[9] and mortality[10]. So, clinical data consistently support the association of ASA with beneficial outcomes in sepsis and pneumonia. However, due to the observational nature of these studies, no cause-effect relationships can be identified. Therefore, these associations instigate the development of hypothesis concerning how anti-platelet therapy could contribute to these observed beneficial effects in sepsis. Two hypothesis on how the immune-enhancing effects of ASA may contribute to any survival benefit in patients with sepsis may be put forward. First, the augmented pro-inflammation caused by low-dose ASA may result in a more effective early clearance of bacteria, resulting in improved survival compared to patients with an initial less pronounced cytokine response[43]. Alternatively, the here described immune-enhancing effects of ASA could also explain the observed beneficial outcomes in sepsis if ASA counteracts sepsis-induced immunoparalysis. Sepsis-induced immunoparalysis is a phenomenon receiving increasing attention, characterized by the inability to clear pathogens and an increased susceptibility to secondary (opportunistic) infections[44]. The immune-stimulating properties of ASA may partially compensate this immune refractant state.

Second, as platelets are increasingly recognized as mediators in inflammation, modulating the immune response with anti-platelet therapy may represent a novel strategy to treat patients with sepsis. Although it is recognized that imbalances in the immune response itself result in the clinical syndrome of sepsis and sepsis-related mortality, until now, anti-inflammatory therapies have failed to improve outcome[45]. The host response in sepsis is complex, with interindividual differences in the inflammatory balance over time. Nevertheless, it is conceivable that modulating the immune response, depending on the inflammatory state, may be of clinical benefit[45]. Although randomized controlled trials have not shown beneficial effects of ibuprofen[46] and lornoxicam[47] in sepsis, this may be due to small sample sizes, patient selection and timing of treatment[45]. Currently, a randomized trial is recruiting patients to evaluate the effects of ASA in patients with severe sepsis or septic shock (Clinical Trials.gov identifier NCT02612480). Alternatively, the observed improved survival of ASA users in sepsis may be based on non-immunologic mechanisms, or despite efforts to correct for baseline differences between ASA and non-ASA users, even be related to other patient characteristics or chance. Therefore, the questions if and how ASA affects outcome in patients with sepsis still require definitive answers.

In this study, we evaluated the effect of clinically relevant antiplatelet treatment schedules on systemic inflammation in a highly reproducible human model. In contrast to bolus administration in previous studies[22], systemic inflammation was elicited with continuous infusion of endotoxin, which resulted in a prolonged inflammatory state and may therefore be more representative of ongoing inflammation as encountered in patients. Although we

have ruled out that adenosine, platelet-reactivity or platelet-leukocyte interactions play a role in ASA/$P2Y_{12}$ inhibitor related immunomodulation, we have not conclusively pinpointed the mechanism through which ASA augments the cytokine response. Nevertheless, human endotoxemia remains an experimental model of inflammation, although well validated, and does not fully reflect the complexity of the pathogenesis of sepsis. Another potential limitation of our study is that, as opposed to patients with sepsis and pneumonia, subjects were young healthy males, limiting generalization to patients. Speculatively, platelet inhibition may have more pronounced effects in patients with activated platelets, as observed in atherosclerosis[48] or Gram-positive sepsis[49].

In conclusion, seven days of treatment with low-dose ASA resulted in an enhanced pro-inflammation during systemic inflammation in humans. Although the addition of ticagrelor to ASA treatment attenuated the TNFα response, ticagrelor and clopidogrel added to ASA did not relevantly alter the ASA-augmented inflammatory response.

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Supplemental Methods

Study design, subjects and study procedures

This prospective randomized open-label, blinded endpoint (PROBE-design) study was performed according to the latest version of the declaration of Helsinki (2008), the Medical Research Involving Human Subjects Act (WMO) and Good Clinical Practice (GCP) and is registered at clinicaltrials.gov identifier NCT01978158.

After approval of the local ethics committee from the Radboud university medical centre, 45 males signed informed consent and were screened for eligibility. Forty healthy volunteers were included as they met all inclusion criteria and none of the exclusion criteria. Inclusion criteria were male sexe, age 18-35 years and no known current medical or psychiatric diseases. Exclusion criteria were history, signs or symptoms of cardiovascular disease, chronic obstructive pulmonary disease or asthma, hemorrhagic diathesis or disorders associated with increased risk of bleeding, pathological bleeding, history of intracranial haemorrhage, history of dyspepsia, quantitative bleeding assessment tool (BAT) score> 3[50], immune deficiency, spontaneous vasovagal collapse, use of any medication, smoking, cardiac conduction abnormalities on electrocardiogram, hypertension (systolic blood pressure >160 mmHg or diastolic blood pressure >90 mmHg), hypotension (systolic blood pressure <100 mmHg or diastolic blood pressure < 50 mmHg), renal impairment (estimated glomerular filtration rate by Modification of Diet in Renal Disease formula < 60 ml/min), liver enzyme abnormalities (ALAT or ASAT > two times the upper limit of reference value), thrombocytopenia (thrombocytes < 150 x 10^9/L), anemia (heamoglobin < 8.0 mmol/L), febrile illness in the week before the endotoxin challenge, hypersensitivity to ticagrelor or any excipients and participation in another drug trial or donation for blood 3 months prior to, until 3 months after the planned endotoxin experiment.

Subjects were randomized to one of four study arms, i.e. ticagrelor and ASA (TA), clopidogrel and ASA (CA), placebo and ASA (PA) or placebo. All subjects received a loading dose of the allocated treatment on day one, and continued with maintenance doses during the seven days preceding the endotoxemia experimental day. Ticagrelor loading dose was 180mg, with a maintenance dose of 90 mg twice daily, clopidogrel loading dose was 300 mg, and with a maintenance dose of 90 mg daily, ASA loading dose was 320 mg, with a maintenance dose of 80 mg daily.

Subjects refrained from alcohol, nicotine and caffeine for 24 hours, and fasted overnight prior to the endotoxemia experiment. After admission to the intensive care research department, an arterial cannula *(Angiocath; Becton Dickinson, USA)* was placed in the radial artery and connected to a blood pressure monitoring system (Edward Lifesciences, Irvine, CA, USA) for blood pressure monitoring and blood withdrawal. A venous cannula provided access for intravenous hydration and endotoxin administration. Heart rate was monitored by a three-lead electrocardiogram and haemodynamic data was recorded from a Philips M50 monitor (Eindhoven, The Netherlands) with an interval of 30 seconds using an in-house developed system. Subjects were prehydrated with intravenous (i.v.) infusion of 1.5L glucose 2.5%/0.45% NaCl in one hour. Purified lipopolysaccharide (LPS, US Standard Escherichia Coli O:113 endotoxin) was obtained from the Pharmaceutical Development Section of the National Institutes of Health (Bethesda, MD, USA). The lyophilized powder was reconstituted in 5 mL saline 0.9% for injection and vortex mixed for 20 min after reconstitution. An i.v. bolus of LPS was administered at a dose of 1 ng/kg at time point 0 hours, continued by a continuous infusion at a dose of 1 ng/kg/ h for 3 hours. Hydration was continued with a rate of 150 ml/h for 6 hours, and 75 ml/h for the rest of the experiment to ensure optimal hydration. Blood was drawn at several time points throughout the experiment. All samples were processed within 3 hours after collection. Temperature was measured using a tympanic thermometer (FirstTemp Genius 2; Covidien, Dublin, Ireland). Endotoxin induced flu-like symptoms (headache, nausea, shivering, muscle and back pain) were scored on a six-point scale (0 = no symptoms, 5 = worst ever experienced), resulting in a total symptom score of 0–25.

Cytokine measurement

Ethylenediaminetetraacetic-(EDTA) anticoagulated blood was immediately centrifuged after withdrawal at 2000 g,4°C for 10 minutes. The obtained plasma was stored at -80°C until further analysis. Plasma concentration of the cytokines Tumor Necrosis Factor (TNF) α, Interleukin (IL)-6, IL-8, IL-10 and IL-1 receptor antagonist (IL-1RA) and the chemokines macrophage inflammatory protein (MIP)-1α, MIP-1β and monocyte chemoattractant protein-1 (MCP-1), also known as C-C motif chemokine ligand 2 (CCL2), were determined batchwise in anticoagulated plasma by a multiplex immunoassay assay according to the manufacturer's instructions (Bio-Plex, BioRad, Hercules, USA and Milliplex, Millipore, Billerica, USA).

Plasma adenosine

Plasma adenosine levels were determined using an in-house developed method as described previously[23]. Blood was drawn with a special syringe system that immediately mixed the blood with a solution that consisted of pharmacological blockers of adenosine formation, transport and degradation at the tip of the syringe in a 1:1 ratio. Haematocrit values in the blocking solution-blood mixture and in whole blood were determined to correct for dilution. The mixture was centrifuged at 2000 g, 4°C for 10 minutes and stored at -80°C until further analysis.

The blocker solution consisted of 40 µM dipyridamole (adenosine transport inhibitor), 10 µM erythro-9-(2-hydroxy-3-nonyl) adenine (EHNA) (adenosine deaminase inhibitor), 10 µM iodotubericidine (ITU) (adenosine kinase inhibitor), 13.2 mM Na2EDTA (disodium ethylenediamine tetraacetate) (inhibits release from platelets and acts as a 5'-nucleotidase inhibitor), 118 mM NaCl, and 5 mM KCl. Plasma adenosine concentrations were determined by high performance liquid chromatography (HPLC). In brief, 36 volumes of plasma were mixed with 1 volume of perchloric acid (70%) and 20 volumes of 0.5M trioctylamine in chloroform, followed by centrifugation (3 min, 13400rpm, RT). Four volumes of supernatant were mixed with 1 volume of chloroacetaldehyde (6x diluted in 1M acetate buffer, pH 4.5) followed by derivatization (60 min, 500rpm, 70 °C). Chloroform was added (3.3 volumes), the mixture was centrifuged (3 min, 13400 rpm, RT), the supernatant was transferred to a HPLC vial and injected. Adenosine was separated by HPLC system (Thermo Scientific, Rockford, USA) using a Polaris C18-A column (150 x 4.6 mm) with gradient elution using eluent A (50 mM $NH_4H_2PO_4$, 5 mM sodium 1-hexanesulfonate monohydrate [pH 3.0], and 2% MeOH) and eluent B (H_2O: ACN: THF; 50:49:1). The retention time was approximately 10 min. Quantification was based on peak areas of the samples and reference standards measured with fluorescence (excitation: 280 nm; emission: 420 nm).

Assessment of P2Y12 inhibition

Specific effectiveness of $P2Y_{12}$ inhibition was determined by the level of phosphorylation *of* vasodilator-stimulated phosphoprotein (VASP) in citrated blood (3,2% sodium citrate, Vacutainer, Becton Dickinson) whole blood using an ELISA-based assay (CY-QUANT VASP/P2Y12, BioCytex, Marseille, France) according to manufacturer's instructions. This test monitors the responsiveness to P2Y12 inhibitors, and P2Y12 inhibition is expressed as platelet reactivity index (PRI%), a value < 50% is considered as adequate platelet inhibition.

Platelet activation and responsiveness

Platelet activation and responsiveness was assessed in citrated (3.2% sodium citrate, Vacutainer, Becton Dickinson) whole blood using flow cytometry within 3 hours. Whole blood (5µL) was added to a mix of hepes-buffered saline, saturating concentrations of FITC-labeled anti-fibrinogen (DAKO, Heverlee Belgium), PE-labeled anti-CD62p (anti-P-selectin; BD Biosciences, San Jose, CA, USA) and PC7-labeled anti-CD61 (anti-glycoprotein IIIa; Beckman Coulter, Brea, CA, USA). Platelet reactivity was assessed by *ex vivo* stimulation with either platelet agonist adenosine diphosphate (ADP; 3.4 and 125 µM; Sigma-Aldrich, Zwijndrecht, The Netherlands) or cross-linked collagen-related-peptide (CRP-XL; 39ng/L or 625ng/L). CRP-XL was a generous gift from Prof. dr. R. Farndale (Cambridge, UK) and was prepared as described previously[51]. After incubation for 20 minutes at room temperature a 500µL fixative solution (0,4% formaldehyde) was added. Samples were directly measured on a FC500 flow cytometer (Beckman Coulter) and data were analyzed using Kaluza® software (Beckman Coulter). Platelets were gated on forward-scatter (FS), side-scatter (SS) and CD61 positivity. Subsequently expression of CD62p, a measure of degranulation, and fibrinogen after ADP stimulation were expressed as median fluorescence intensity (MFI).

Leukocyte and platelet counts

Analyses of leukocyte counts, differentiation and thrombocyte counts were performed in EDTA anticoagulated blood using routine analysis methods also used for patient samples (flow cytometric analysis on a Sysmex XE-5000).

Statistical analysis

Data are expressed as a mean ± SEM or as median [interquartile range], according to their distribution. Data were tested for normality using the Shapiro-Wilk test. To evaluate the effect of platelet inhibitors on cytokine responses, PRI and activity and responsiveness, repeated measures two-way ANOVAs (interaction term of group and time) were performed between specific pairs of groups: 1. To evaluate the effects of ASA, placebo-ASA was compared to placebo 2. To evaluate the effect of clopidogrel or ticagrelor added to ASA, placebo-ASA was compared to clopidogrel-ASA and ticagrelor-ASA. 3. In case of an effect of ticagrelor or clopidogrel added to ASA, a comparison of clopidogrel-ASA with ticagrelor-ASA was made to assess for differences between these P2Y$_{12}$ inhibitors.

Baseline values of prostaglandins, plasma adenosine and VASP-P platelet reactivity index were compared with Students t-test or Mann-Whitney test. Differences in changes over time were analyzed with Friedman test with post-hoc Dunn's Multiple Comparison Test (thromboxane B and VASP-P) or Wilcoxon signed rank test (adenosine). Haemodynamic parameters, temperature, symptom scores, platelet and leukocyte counts were analyzed for differences between all four groups using a repeated measures two-way ANOVA (interaction term of group and time). Correlation between platelet responsiveness and plasma cytokine levels were calculated using Pearson's correlation. All statistical analyses were performed using GraphPad Prism version 5.03 (GraphPad Software, San Diego, CA, USA). A two-sided p value <0.05 was considered statistically significant.

Supplemental Table 1.

Condition	Ref	Patient group	Study type	N	Comparison	Analysis	Primary outcome	Effect
Sepsis and systemic inflammatory syndrome	1	ICU patients with severe sepsis or septic shock	Retrospective cohort study	979	ASA vs no ASA	Logistic regression	In-hospital mortality	adjusted odds ratio 0.57 (0.39-0.83)
	2	Surgical ICU patients with sepsis	Retrospective cohort study	886	ASA vs no ASA	Logistic regression	In-hospital mortality	adjusted odds ratio 0.57 (0.39-0.83)
	3	ICU patients with first time sepsis or SIRS	Retrospective cohort study	5523	ASA vs no ASA	Logistic regression (total cohort) and propensity analysis (n=270)	In-hospital mortality	Logistic regression: absolute risk reduction of 6.2% (3.5-9.5%) Propensity analysis: absolute risk difference -14.8% (-18.9 to -8.6%)
	4	Hospital patients with sepsis	Nation-wide population based cohort study, with nested case-control study	683421	Antiplatelet agents vs no antiplatelet agents*	Logistic regression and case-control design (n=372748)	mortality	Logistic regression: adjusted odds ratio 0.82 (0.81-0.83) Case-control: adjusted odds ratio 0.78 (0.76-0.79)
	5	Patients with pneumonia and septic shock	Prospective observational study	188	ASA combined with macrolide vs no ASA-macrolide	Logistic regression	30-day mortality	Hazard ratio 0.25 (0.08-0.82)
	6	ICU patients with sepsis	Prospective observational study	972	Antiplatelet therapy vs no antiplatelet therapy†	Propensity analysis (n=300)	30-day mortality	Hazard ratio 1.21 (0.79-1.84)
Pneumonia	7	Patients with community acquired pneumonia	Prospective observational study	1005	ASA vs no ASA	Cox regression and propensity analysis	30-day mortality	Regression: Hazard ratio 0.43 (0.25-0.75) Propensity analysis; non-aspirin group has a hazard rate of 2.07 (1.08-3.98)
	8	Patients with community acquired pneumonia	Retrospective cohort study	224	Anti-platelet drugs vs no anti-platelet drugs‡	Logistic regression	Need for ICU admission	Adjusted odds ratio cohort: 0.32 (0.1-1.0) age matched: 0.19 (0.04-0.87)

Cohort studies evaluating the association between thrombocyte aggregation inhibitors and outcome in patients with sepsis and pneumonia. * Aspirin, clopidogrel and ticlopidine, †Acetylsalicylic acid, clopidogrel, dipyridamole.

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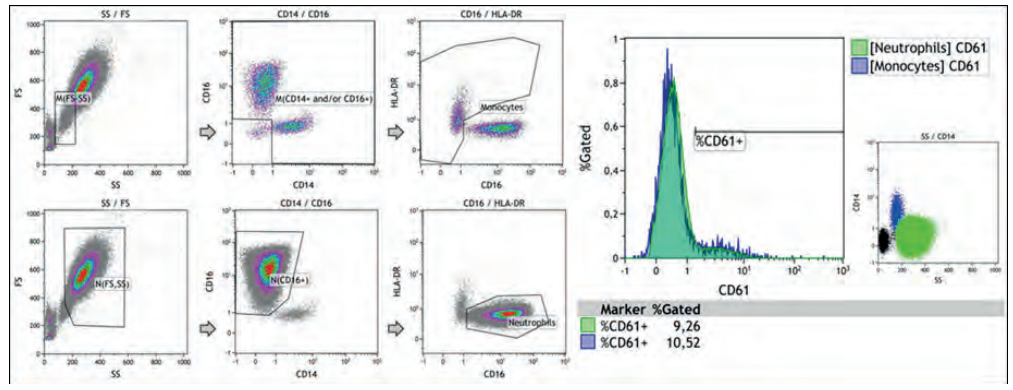
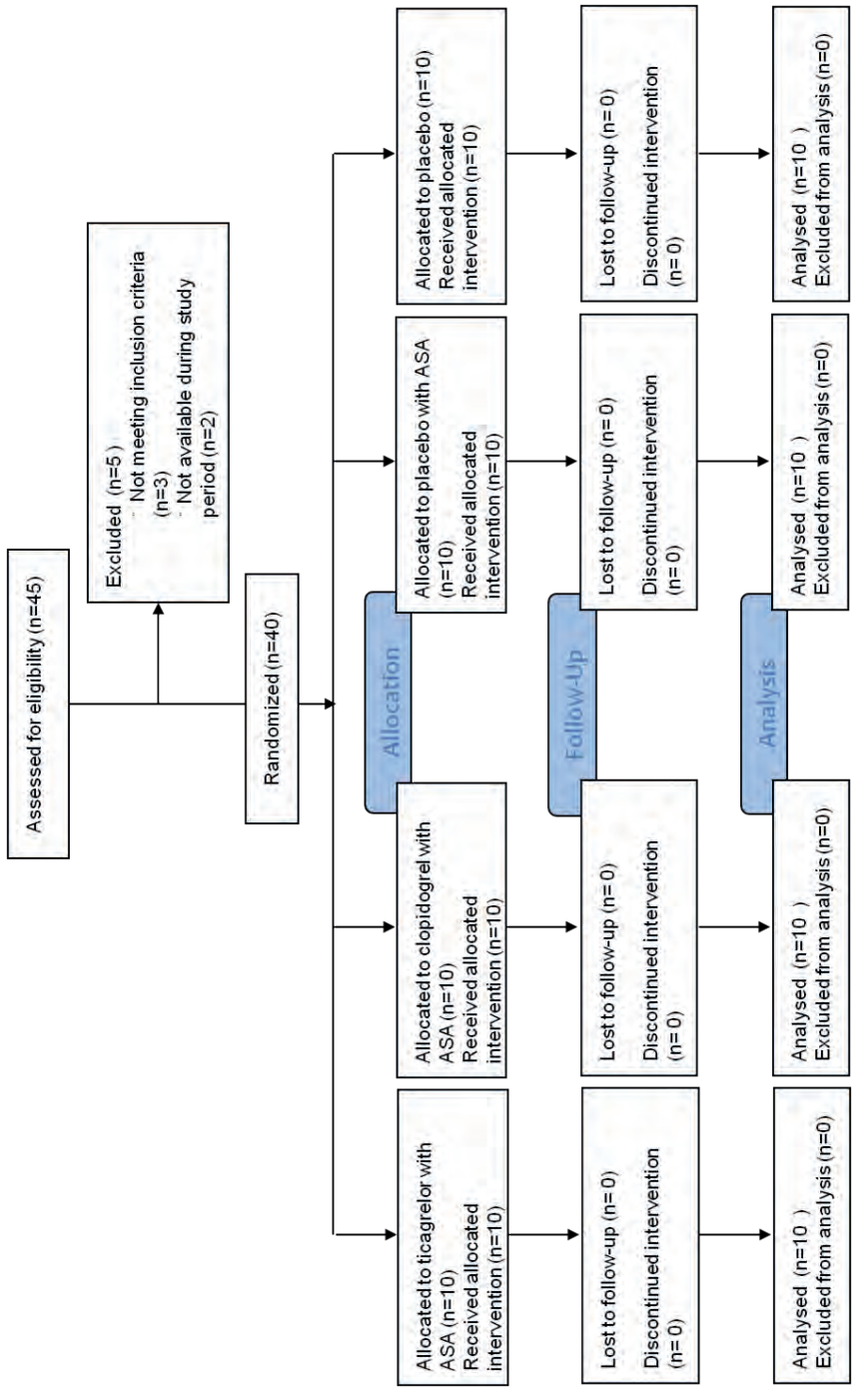
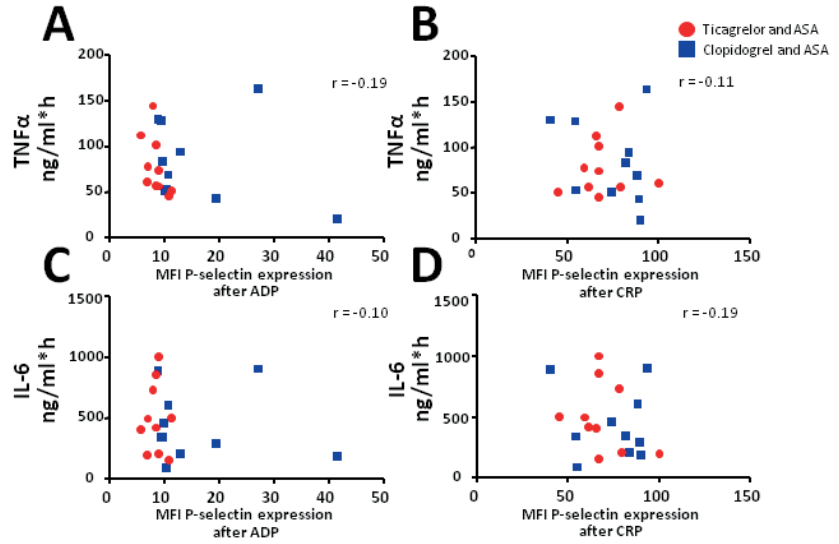


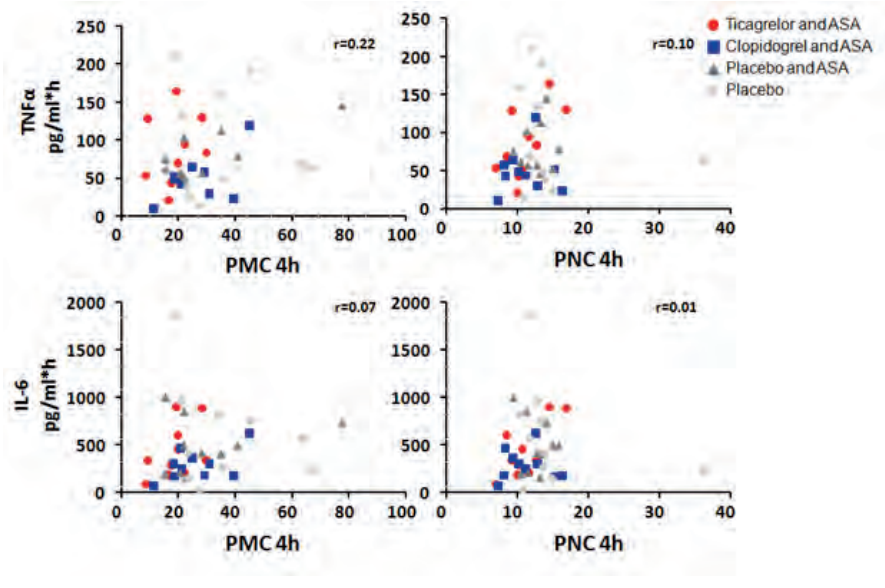
Supplemental Figure 1. Gating strategy platelet-leukocyte complexes. Gating strategy for platelet-monocyte and platelet-neutrophil complex formation in a representative sample. Monocytes (blue) were selected based on size (forward-scatter; FS), granularity (side-scatter; SS) and expression of CD16, CD14 and HLA-DR as shown in the upper scattergrams. Neutrophils were gated based on size (FS), granularity (SS) CD14, CD16 and HLA-DR as shown in the lower scattergrams. The markers were chosen to distinguish neutrophils and monocytes during experimental human endotoxemia. Platelet interaction was assessed using the % of CD61-positive events shown in the right overlay graph. An example of the different populations of monocytes (Blue) and neutrophils (green) are shown left in the scattergram. Kaluza Analysis 1.5 (Beckman Coulter, Brea, CA, USA) was used for analysis.



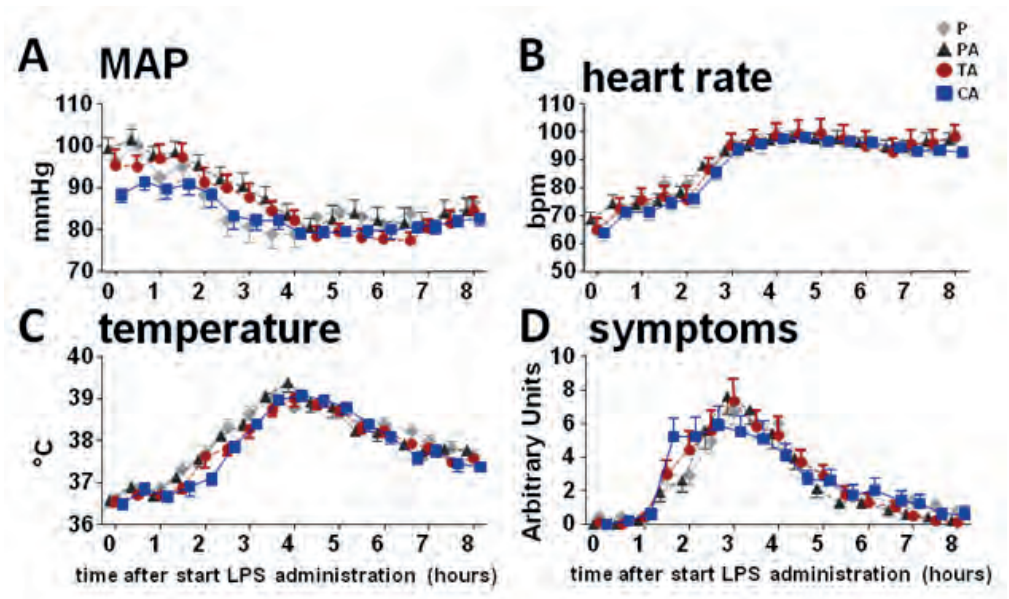
Supplemental Figure 2. CONSORT Flow chart of study inclusion and follow up



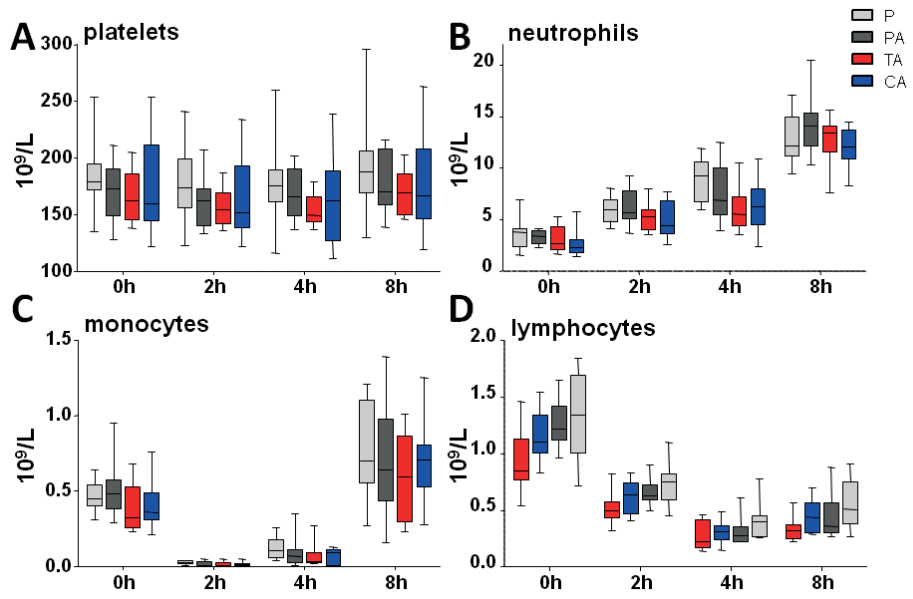
Supplemental Figure 3. Correlations between tumor necrosis factor (TNF)α (A and B) and interleukin-6 (C and D) and platelet reactivity expressed as P-selectin expression upon stimulation with high doses of collagen related peptide (CRP-XL) (A and C) or adenosine diphosphate (ADP) or at time of endotoxin administration. Cytokine production is expressed as the area under the concentration-time curve (ng/ml*h). Pearson's correlation coefficients were not significant. ASA: acetylsalicylic acid.



Supplemental Figure 4. Correlations between tumor necrosis factor (TNF)α and interleukin-6 and platelet monocyte complexes (PMC) or platelet neutrophil complexes (PNC). Cytokine production is expressed as the area under the concentration-time curve (ng/ml*h). Pearson's correlation coefficients were not significant. ASA: acetylsalicylic acid.



Supplemental Figure 5. (A) Mean arterial pressure (MAP), (B) heart rate, (C) temperature and (D) symptom score during endotoxin-elicited systemic inflammation. Endotoxin administration started at t=0 hours and continued until t=3 hours. Data are expressed as mean and SEM. MAP decreased over time, with a concurrent increase in heart rate and temperature (1-way ANOVA in P group, p<0.0001 for all parameters). There was a small difference in MAP between groups (2-way ANOVA p=0.04), although post-hoc analysis did not point out what the discrepant group or time point was. There was no effect of study treatment on heart rate, temperature and symptom score. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.



Supplemental Figure 6. (A) platelet-, (B) neutrophil-, (C) monocyte-, and (D) lymphocyte counts during endotoxin-elicited systemic inflammation. Endotoxin administration started at t=0 hours and continued until t=3 hours. Data are expressed in box plots with minimal and maximal observed values. Platelets, monocytes and lymphocytes decreased temporarily due to endotoxemia (1-way ANOVA in P group, p<0.0001 for all parameters), without any effect of study treatment on cell counts. P: placebo, PA: placebo and acetylsalicylic acid, TA: ticagrelor and acetylsalicylic acid, CA: clopidogrel and acetylsalicylic acid.

Chapter 10

Summary and general discussion

Summary

The introduction of combination antiretroviral therapy (cART) has revolutionized HIV care by strikingly improving morbidity, mortality and transmission[1-4]. Although drug toxicity remains a concern, the clinical advantages of viral suppression are clear[5]. The extended choice of different antiretroviral drugs from nucleoside reverse transcriptase inhibitors (NRTI), protease inhibitors (PI), nonnucleoside reverse transcriptase inhibitors (NNRTI), and integrase inhibitors (INSTI) has increased possibilities for patients suffering from adverse effects of cART. These therapeutic options have transformed HIV from a deadly condition, into a chronic manageable disease[6-8]. Still, multi-morbidity is common[6,7] and people living with HIV (PLHIV) remain at increased risk for non-AIDS related comorbidities such as cardiovascular disease and cancer[6,9-11]. The clinical necessity of tackling these comorbidities is important as HIV cure remains a distant prospect[12]. A better understanding of the persistent immune defects and inflammation is important as these are associated with increased risks for cancer and cardiovascular disease (CVD)[11] as well as HIV persistence[13-15]. Furthermore, the complex interplay between inflammation and coagulation, is known to play a crucial role in the development of cardiovascular diseases[16]. This complex interplay has been termed thrombo-inflammation.

In this thesis, multiple aspects of thrombo-inflammation in PLHIV were studied. In **Chapters 2-6**, data generated from a Dutch HIV-cohort of 217 PLHIV with prolonged viral suppression are presented. This cohort is part of the Human Functional Genomics Project (HFGP)[17]. In **Chapters 7-9**, additional studies are described to better understand thrombo-inflammation in PLHIV. This final chapter covers a summary of our findings and an elaboration on future perspectives.

In **Chapter 2,** we showed the presence of persistent inflammation in PLHIV with prolonged viral suppression. Since PLHIV showed increased concentrations of circulating inflammatory markers compared to uninfected controls. By using an integrative approach of functional and transcriptional analyses, the functional phenotype of circulating immune cells were analyzed. We found that stimulation of circulating immune cells resulted in a sustained increased monocyte cytokine responsiveness, most notably for IL-1β, in PLHIV compared to uninfected controls. This increased responsiveness was associated with persistent inflammation, suggesting a functional link between changes in the innate immune cell function and systemic inflammation in PLHIV. The observed intrinsic pro-inflammatory phenotype of monocytes in PLHIV was stable over time and associated with upregulation of pro-inflammatory pathways, most notably the interleukin (IL)-1β pathway. This was confirmed using a second age-sex matched control group in which we also performed transcriptome analysis. Upregulated genes were intracellular signaling proteins and inflammasome related molecules (e.g., NLRP3, STAT1), cytokines (e.g., IL1B, CCL2, MMP9, IL1RN), and pattern-recognition receptors (e.g. TLR2, TLR4, TLR7 and NOD2), indicating the broad upregulation of inflammatory pathways in monocytes of PLHIV on stable cART.

Monocytes are known to play a crucial role in the development of atherosclerosis[18,19]. Monocytes can be reprogrammed after exposure to endogenous and microbial structures by changes at the epigenetic level, a process termed "trained immunity"[20,21]. This trained immunity phenotype renders monocyte with a long-term enhanced functional state, showing a stronger proinflammatory response (e.g. IL-6 and IL-1β production) to a second stimulus[22]. Trained immunity is known to rely on epigenetic modifications such as histone modification and DNA methylation[22-24] but also metabolic reprogramming[25]. Clinical implications of trained immunity have been shown in multiple diseases such as cardiovascular disease and diabetes[26-29]. Our data indicate a similar trained immunity phenotype in PLHIV with a stable increased cytokine production capacity with augmented pro-inflammatory pathways.

A recent study by Bowman et al[30] showed that monocyte-derived macrophages from PLHIV, were pro-atherogenic with pro-inflammatory transcriptional phenotypes and increased foam cell formation, both hallmarks of trained immunity[19]. This phenotype was induced through PLHIV serum and linked to lipid alteration, a further indication to trained immunity, as oxidized Low-density lipoprotein (LDL) is known to induce a trained immunity phenotype. Another strong inducer of trained immunity is (1→3)-β-D-glucan (β-glucan)[24], a fungal cell wall product[31]. In this thesis we show that concentrations of β-glucan in serum are increased in PLHIV compared to controls[32-34]. Furthermore, we could associate the presence β-glucan in serum of PLHIV to a pro-inflammatory phenotype of monocytes. HIV is associated with impaired intestinal integrity[35,36] and different studies have reported increased circulating LPS concentrations in PLHIV[35,37]. Increased microbial translocation may also lead to increased plasma β-glucan concentrations[32,34,38]. The immune effects of LPS and β-glucan differ, as also shown by our *in-vitro* data. Whereas LPS exposure induces tolerance, even at very low concentrations, β-glucan induces a pro-inflammatory phenotype[21,23,39]. These observations warrant further study in PLHIV, focusing on gut barrier dysfunction and the gut mycobiome, a compartment of the microbiome, which is overlooked in the general population, but also in special groups such as PLHIV[40].

The gastrointestinal tract is a crucial for HIV replication and possibly for persistence of the HIV reservoir[41]. Local disruption of the intestinal Th17 and regulatory T-cells (Tregs) balance may have major consequences for the maintenance of gut barrier integrity and function[42]. A disrupted intestinal mucosal barrier may Increase exposure to endogenous and microbial structures leading to epigenetic modification and resulting in trained immunity[21]. Pharmacological interventions targeting epigenetic modification have been

used in PLHIV, especially in attempts to reduce the HIV reservoir and to achieve (functional) cure[43]. Treatment with these epigenetic modifying drugs such as histone deacetylase inhibitors (HDACi) reduces high-sensitivity C-reactive protein (hsCRP) and IL-1β expression in peripheral blood mononuclear cells (PBMCs)[44] and reduces IL-1β production upon LPS stimulation in whole blood [45]. However, these HDACi have not been used to broadly target persistent inflammation.

Besides HDACi, statins[46-48], aspirin[49] and other anti-inflammatory drugs have been used to reverse persistent inflammation with limited success[9]. In **Chapter 2**, we showed the importance of the IL-1β pathway and thereby also as a possible therapeutic target for persistent inflammation in PLHIV and HIV-related cardiovascular disease and atherosclerosis[50]. The CANTOS (Canakinumab Anti-Inflammatory Thrombosis Outcome Study) trial, which that was carried out in non-HIV infected subjects with a recent cardiovascular event and residual inflammation showed that inhibition of IL-1β via subcutaneous injection was associated with a reduction in the incidence of subsequent cardiovascular events[51]. A subsequent trial using the orally administered anti-inflammatory drug colchicine showed a similar reduction in cardiovascular events in patients with stable and recent coronary disease[52]. In contrast, the immunosuppressive drug methotrexate was not successful in reducing CVD, supporting thereby the importance of IL-1β in CVD[53]. In a pilot study, canakinumab reduced atherosclerotic inflammation, measured by Positron Emission Tomography (PET)-FDG scanning, in PLHIV at high-risk for CVD[54] which further supports the importance of IL-1β pathway in HIV.

In addition, an exploratory analysis of the CANTOS trial found a dose-dependent reduction in incident lung cancer and fatal lung cancer for IL-1β inhibition, with those with increased IL-6 at highest risk[55]. Inflammation in the tumor microenvironment mediated by IL-1β is linked to cancer progression, and metastases[56,57]. Smokers and those with the most pronounced reduction in inflammation upon IL-1β inhibition gained the most benefit[55]. As cancer is another major cause of morbidity in long-term treated PLHIV[5], IL-1β inhibition could provide an interesting target for immune based therapy. Orally administrated treatments that specifically inhibit the IL-1β pathway, such as inflammasome inhibitors or colchicine may form an attractive alternative to subcutaneous injections[52,58].

In **Chapter 3**, we focused on the circulating immune cell composition by examining 108 different cell types in PLHIV and comparing these with healthy controls. Despite being on long-term suppressive cART, PLHIV exhibited an expansion of cluster differentiation (CD)8 cells and lower proportions of CD4+ T cells and an immune cell profile characterized by an increase in proliferating memory and effector CD4 and CD8 T-cells. The number of circulating T-helper 17 cells (Th17) and pro-inflammatory Th17-like (CC chemokine receptor 6 (CCR6)+) regulatory (Treg) T-cells, were increased. The relationship between pro-inflammatory Th17 cells and suppressive Tregs must remain balanced to preserve functional immunity. Altered ratios have been described in untreated HIV (lower Th17/Treg), autoimmune disease and cancer (higher Th17/ /Treg)[59-61]. In line with Th17 levels, PLHIV showed increased peripheral blood Th17/Treg ratios, which is apparent contrast to earlier reports of massive depletion of Th17 cells from the peripheral blood and the gut[59], a process that is partially reversed by cART[62,63]. IL-1β and IL-6 are critical cytokines driving T-cell differentiation into Th17 cells[64]. Indeed, the pro-inflammatory phenotype (described in **Chapter 2**) correlated with Th17 frequencies, as circulating IL-6 correlated with Th1y frequencies.

These changes in cell subsets also translated into functional changes in adaptive immunity with reduced interferon (IFN)-γ levels upon stimulation with *C. albicans* and *M. tuberculosis*. While Th17-subsets were increased, we found no effect on *ex vivo* IL-17 and IL-22 production by peripheral blood mononuclear cells. Parameters of the HIV reservoir (e.g. cell associated HIV DNA and RNA) correlated with IFNy production and with several circulating immune cell subsets, most notably, inflammatory Treg subsets, Th17 cells, central memory CD4 cells and markers of T-cell expansion. Besides the reservoir, CMV co-infection, CD4 nadir, age, sex and smoking also contributed to the T-cell repertoire in PLHIV. This comprehensive overview of the peripheral immune cell composition showed a dysregulated immune profile in PLHIV. The observed link with the HIV reservoir will be combined with transcriptomic and proteomic analyses in the future to pinpoint new culprits in HIV persistence. Such hypothesis generating analyses could open new avenues in HIV cure research.

Still, Th17 cells in the peripheral blood poorly reflect mucosal Th17 numbers [65]. As such, we cannot exclude that altered Th17 recruitment leading to mucosal Th17 depletion is still present despite suppressive cART. This depletion could lead to an altered mucosal immunity and possibly lead to increased microbial translocation. Indeed, intestinal fatty acid binding protein (IFABP), a marker of intestinal integrity, was increased in our cohort and correlated with Th17 cell numbers. Furthermore, the gut homing chemokine (CCL20) correlated with Th17 cell frequencies, underlining the importance of gut immune homeostasis in PLHIV. Future studies are warranted to investigate the link between peripheral and mucosal Th17 cells in long-term virally suppressed PLHIV.

In **Chapter 4**, the occurrence of somatic mutations driving clonal hematopoiesis in PLHIV are described. Somatic mutations in HIV-uninfected individuals have been implicated in the risk and development of cardiovascular disease as well as hematological (pre)malignancies[66]. Targeted sequencing of known genes involved in clonal hematopoiesis in both the HIV-cohort, in another HFGP cohort, namely a cohort of subjects at risk for CVD (300-OB) and uninfected population controls, enabled us to explore the occurrence of clonal hematopoiesis in PLHIV on stable cART. The prevalence of clonal hematopoiesis adjusted for age was

increased and clone size was larger in PLHIV compared to population controls. Moreover, older age, low CD4 nadir and high CD4/CD8 ratio were associated with clonal hematopoiesis within PLHIV. A recent study by Bick et al (not yet peer-reviewed) also reported increased clonal hematopoiesis in PLHIV[67]. Although an association with cART duration as well as with age and coronary artery disease, no correlations with other HIV-related parameters such as CD4 levels, HIV medication, HIV reservoir parameters were found. Still, our studies suggest that that the risk for somatic mutations is increased PLHIV and that HIV-specific factors may play a role.

Signature analysis of the found mutations showed a unique mutational signature in PLHIV which has been linked to reactive oxygen species (ROS) in cancer[68]. Although, sample size limited the ability to draw strong conclusions, PLHIV with prior exposure to zidovudine, a NRTI, were exclusively responsible for this ROS-associated mutational signature. Zidovudine is well-known for its hematological adverse effects. Mitochondrial dysfunction is a hallmark of NRTI toxicity and leads to oxidative stress (i.e. ROS)[69]. Consequently, CHDMs with a ROS mutational signature could possibly be induced by NTRI exposure. The observation by Bick et al that cART duration is linked to clonal hematopoiesis could support this hypothesis[67]. However, future studies are warranted to study the effect of NRTIs on CHDM development.

In virally suppressed PLHIV, the HIV-1 CA-DNA roughly equals total integrated parvoviral DNA, while HIV-1 CA-RNA is associated with HIV-1 transcriptional activity [70]. The size of the reservoir measured by CA-DNA is associated with timing CD4 nadir and duration of cART treatment[13]. Although both parameters of the HIV reservoir strongly intercorrelate, HIV CA-RNA was increased in CHDM carriers and HIV CA-DNA was not. This discrepancy between CA-RNA and CA-DNA suggests an effect on viral transcriptional activity[70]. Although the CA-RNA difference disappeared after correction for CD4 nadir and age, the ratio of HIV-1 CA-RNA to CA-DNA, which represents viral transcription activity, was increased in CHDM carriers, independent of CD4 nadir, age and CD4/CD8 ratio.

HIV transcription is known to rely on epigenetic modifications such as DNA methylation[71-73]. Modulating transcriptional activity is a major field of research in HIV cure, especially those who pursue a shock-and-kill (or Kick-and-kill) strategy[74]. This strategy involves inducing HIV-1 transcription and replication and thereby reversing latency (shock), followed by an immune-mediated recognition and destruction of infected cells (kill). As CHDMs in the epigenetic regulators *TET2* and *DNMT3A* have been linked to changes in DNA methylation[75], increased HIV transcription activity in CHDM carriers could be a result of aberrant DNA methylation or altered gene expression linked to clonal hematopoiesis[75]. Recently, the integration site of HIV-1 DNA into the genome (e.g. distance to transcriptional start site and accessible chromatin marks)[73] has been linked to spontaneous control in HIV elite controllers. The observed differences in transcriptional activity in CHDM carriers could , therefore, also be the result of modulation of these integration sites. Future studies on the effect of CHDMs on epigenetic marks and integration sites of HIV-1 DNA are warranted.

It has been suggested that clonal hematopoiesis increases cardiovascular risk through augmentation of inflammatory pathways. While, we found that CHDM-carriers had increased markers of coagulation compared to non-carriers, we did not observe such correlation for inflammation. Importantly, this procoagulant state in CHDM-carriers was independent of age and CD4 nadir. Therefore, clonal hematopoiesis could serve as a novel risk factor for non-AIDS related comorbidities in PLHIV.

Previous studies have shown that D-dimer concentrations are increased in PLHIV and are linked to mortality and the occurrence of cardiovascular disease[76]. D-dimer is a biomarker of *in vivo* coagulation, depending on coagulation factors and signals that provoke coagulation, such as inflammation and endothelial activation. To date, published data on coagulation capacity in PLHIV are conflicting[77-79]. The capacity of plasma to form thrombin is a critical determinant of *in vivo* plasmatic coagulation[80]. Thrombin generation can be measured *ex vivo* to determine coagulation capacity in a standardized setting and has been used as a diagnostic tool for hypo- and hypercoagulability states[81]. In **Chapter 5**, we describe thrombin generation capacity in our 200HIV cohort and explore factors associated with these functional coagulation assays. In line with earlier studies, D-dimer concentrations were increased in PLHIV compared to uninfected controls. At the same time, thrombin generation was slightly reduced in the PLHIV, after correction for age, sex and antiretroviral regimens. However, abacavir-use was independently associated with increased thrombin generation. This observation may shed light on the reported increase in thrombotic events during abacavir-use[82,83]. Furthermore, we showed that D-dimer concentrations were more associated with inflammation than plasmatic coagulation capacity. Overall, we concluded that the increased D-dimer concentrations found in PLHIV are most likely the result of increased exposure to triggers that propagate coagulation, such as inflammation and endothelial cell activation, rather than an intrinsic activation of the coagulation system.

The role of platelet mitochondrial dysfunction has been increasingly recognized in thrombosis, immunoregulation and age-related disease[84]. Mitochondrial toxicity is a well-known complication of cART. The older generation NRTI's, such as zidovudine and stavudine, were associated with profound mitochondrial toxicity, but mitochondrial toxicity may still occur with more recent NRTIs[85].

Mitochondria are essential in maintaining platelet health and lifespan, as recently reviewed[86]. Platelet dysfunction has been reported widely in PLHIV[49,87-93], but the link

between platelet function and platelet mitochondria has received limited attention[93]. In **Chapter 6**, we show that platelets of PLHIV on long-term cART have reduced platelet mitochondrial content ($mtDNA_{pl}$). These lower $mtDNA_{pl}$ levels were associated with platelet mitochondrial dysfunction and reduced energy supply. These mitochondrial perturbations were linked to reduced *ex vivo* platelet reactivity showing the functional consequences of HIV-associated mitochondrial dysfunction. This mitochondrial dysfunction was also linked to markers of persistent inflammation (e.g. sCD163).

Given the key role of platelets and mitochondria in the pathophysiology of cardiovascular events and other age-related conditions[84,86], platelet mitochondria may be an attractive target to reduce long-term complications. Traditionally cART consists of a backbone of two NRTIs and one NNRTI, PI or INSTI. Recently, regimes with only one NRTI or even no NRTI have been introduced[94]. Future studies are needed to explore whether these new NRTI sparing regimens are associated with preserved platelet mtDNA and less platelet dysfunction.

The next chapters are focused on the role of platelets and thrombo-inflammation in HIV and were not performed within the HIV-cohort of the HFGP project. Mounting evidence supports the notion that platelets play an important role in inflammation[16].

A previous cross-sectional study from our group found that individuals on a raltegravir-based regimen showed reduced platelet reactivity and platelet-monocyte complexes compared with those on a non-INSTI-based regimen[90]. **Chapter 7** reports the results of a randomized controlled trial studying the effects of switching cART to a regimen based on the integrase inhibitor raltegravir. We show that switching PLHIV on a non-INSTI regimen to a raltegravir-based regimen for ten weeks did not reduce platelet reactivity, platelet-leukocyte aggregation, monocyte activation or T-cell dysfunction. Different explanations for this difference can be considered. First, the follow-up period of ten weeks in the present study is relatively short, whereas most patients in the cross-sectional study were on the same cART longer than three months. Still, the life-span of platelets is short (<10 days) and several studies have shown effects of raltegravir intensification on viral replication and plasmatic coagulation within three months[95,96]. Nevertheless, platelets are produced by megakaryocytes and we cannot exclude an effect of cART on megakaryocytes, possibly via epigenetic modification or modifying mitochondrial function. Subsequent alterations in platelet reactivity may take longer and require a prolonged treatment switch. Second, cross-sectional studies are prone for selection bias and confounders. Due to a relatively small sample size, the aforementioned cross-sectional study was not able to correct for possible confounders in a multivariate regression model or perform subgroup analyses.

Platelets may modulate immune function in several ways, among others by forming aggregates with immune cells, through P-selectin (on platelets) and P-selectin glycoprotein ligand (PSGL)-1 binding (on immune cells), thereby altering their function. Platelets are mostly considered pro-inflammatory, although binding to regulatory T-cells may also result in reduced inflammation. In **Chapter 8**, we show that platelet microparticles (PMP) can bind to a specialized subset of Th17-like progenitor cells in the Treg compartment and thereby inhibit their differentiation into potentially pathogenic effector cells. Compared to HIV-uninfected individuals, binding of plasma-derived microparticles from PLHIV to Tregs is decreased, suggesting reduced inhibition of differentiation of Tregs into IL-17 and IFN-γ producing cells.

Various drugs have been used to inhibit and modulate platelet function for secondary cardiovascular prevention[97]. Low-dose acetyl salicylic acid (ASA or aspirin), commonly known as aspirin, is traditionally used for platelet inhibition, but this is increasingly combined or replaced by $P2Y_{12}$ inhibitors, such as clopidogrel and ticagrelor [98]. In **Chapter 9**, we evaluated the effects of clinically relevant dosages of aspirin, ticagrelor, and clopidogrel on systemic inflammation during human endotoxemia, a well-known model to study *in vivo* inflammation in humans[99]. While the effect of $P2Y_{12}$ inhibition on inflammation was limited, a seven-day course of low-dose aspirin profoundly enhanced the pro-inflammatory response (e.g. TNFα), most likely through platelet-derived prostaglandin E2 via Cyclooxygenase (COX)-1 inhibition. Aspirin is generally known for its anti-inflammatory properties and has therefore been used to treat HIV-related persistent inflammation, although with limited success[100]. Our data offers an explanation why low-dose aspirin did not result in a detectable anti-inflammatory effect in earlier studies[100] and may even increase inflammation in inflammatory conditions[101].

General discussion and future perspectives

In this thesis immune and coagulation disturbances associated with HIV are described. Inflammation and coagulation are tightly linked and 'thrombo-inflammation' is increasingly recognized to play an important role in different conditions, including atherosclerosis and tumorigenesis[18,102]. These insights have provided a rationale for the development of anti-inflammatory treatment strategies in the prevention of cardiovascular events as well as immune-based therapy for different malignancies. In line with this reasoning, the novel insights described in this thesis do not only increase our understanding of HIV pathobiology but may also provide leads for the development of immune-based therapies to prevent the long-term complications of HIV.

This thesis shows that the regulation of the immune and coagulation system in long-term treated PLHIV is disturbed at several levels. First, within the innate immune compartment, monocyte responsiveness was increased with activation of the IL-1β pathway. Our data indicate a similar trained immunity phenotype in PLHIV with a stable increased cytokine production capacity with augmented pro-inflammatory pathways. Clinical implications of trained immunity have been shown in multiple diseases such as cardiovascular disease and diabetes[26-29]. Trained immunity is known to rely on epigenetic and metabolic reprogramming[22-24] and can be induced by infection, vaccines, pathogen-associated molecular patterns (PAMPs) but also cytokines and oxidized LDL[22,103]. HIV is frequently associated with impaired intestinal integrity and we postulate that leakage of β-glucan in the circulation, the most potent inducer of trained immunity known to date[23], contributes to the trained immunity phenotype in PLHIV. Serum concentrations of β-glucan were indeed increased in our PLHIV cohort, as also previously reported by others[32-34]. Future studies are warranted to investigate also the epigenetic and metabolic processes underlying the observed trained immunity phenotype in PLHIV.

Second, we found disturbances in the adaptive immune system, despite viral suppression. HIV primarily infects CD4+ T-cells and a lower proportion of CD4+ T cells alongside an expansion of CD8 cells were found in this cohort. Within CD4+ cells, we found higher levels of Th17 subsets with increased Th17/Treg ratios in PLHIV. Increased Th17/Treg ratios have been linked to cardiovascular disease and atherosclerosis[60]. Notably, the proportion of Th17-like CCR6+ Tregs was increased as well, further suggesting a pro-inflammatory state. In contrast, IL-17 production upon *ex vivo* stimulation was not different between PLHIV and controls, suggesting that the functional capacity of these cells is compromised. These cells may home to tissues, like the gut, where reduced number of Th17 cells are found in PLHIV, also during long-term cART[42]. Th17 cells are important to maintain intestinal integrity. Reduced mucosal immunity and increased intestinal permeability may contribute to the higher β-glucan levels in PLHIV, as found by us and reported by others[32,33]. Whether IL-17 disbalance contributes to development of cardiovascular disease is debated[104]. Our data suggest that normal T-cell renewal is disturbed in PLHIV with less naïve cells and an enrichment of terminally differentiated T cells with signals of clonal expansion. Correspondingly, we observed a reduction in *ex vivo* IFN-γ production capacity among PLHIV. This functional defect was associated with parameters of the HIV reservoir and could play a role in HIV persistence[105,106] and defective vaccination responses seen in PLHIV on stable cART[107].

Third, we describe different functional abnormalities in platelets and the plasmatic coagulation system. As mentioned above, the function of the coagulation and immune systems are tightly linked and both platelets and thrombin are not only important in bleeding and thrombosis, but also in the regulation of the different parts of the immune system[16,108,109].

A novel insight in this link is described in **Chapter 7**. We report that platelet microparticles inhibit further differentiation of Tregs into Th17 producing Tregs. Our finding that platelets microparticles from PLHIV showed reduced capacity to inhibit this process may underly the increased proportion of pro-inflammatory Th17-like CCR6+ Tregs that we found in PLHIV. Changes in platelet function may also contribute to thrombo-inflammation. In this thesis, we report that the mitochondrial content ($mtDNA_{pl}$) of platelets is reduced and that these lower $mtDNA_{pl}$ levels are associated with platelet mitochondrial dysfunction and reduced energy supply. This mitochondrial dysfunction appeared to be long-lived as prior zidovudine users seemed to be more at risk for mitochondrial depletion. Since, platelets are short-lived (<10 days), an effect on megakaryocytes is plausible. Given that megakaryocytes produce the majority of circulating MPs[110], mitochondrial alterations[86] (and possibly epigenetic modifications[111]) of megakaryocytes could affect anti-inflammatory properties of microparticles in PLHIV. Regarding the plasmatic coagulation, thrombin generation was reduced in PLHIV despite increased plasma D-dimer concentrations. In summary, in our cohort of PLHIV on long-term cART, we did not find a procoagulant state in PLHIV. Although we corroborated multiple links between coagulation and inflammation in **Chapters 4-6** and **7-9**, disturbances within the coagulation pathway itself seem to be limited compared to inflammatory processes.

Developing directed therapies for these disturbances may improve the care for PLHIV. The next step is extrapolating our results towards the development of new treatment strategies. Currently HIV-care is shifting focus towards reducing AIDS and non-AIDS related comorbidities. Paramount is the early initiation of cART, mitigating most of the increased mortality associated with HIV[5]. However, this very early cART-strategy should be accompanied by efforts to minimize cART toxicity.

Regarding this toxicity, the European AIDS Clinical Society (EACS) guidelines suggest to modify cART regimens in people at increased cardiovascular risk (10 year CVD risk >10%)[112]. These guidelines recommend replacing zidovudine and abacavir to other NRTIs or replacing a traditional cART regimen to a NRTI sparing regimen[112]. Abacavir has been linked to a higher risk for cardiovascular events[82,113]. We found that platelet reactivity was similar between abacavir users and non-abacavir users supporting findings of some researchers[114,115] but disputing findings of others[114-118]. A recent paper hypothesized that abacavir induces endothelial cell activation, which may lead to both platelet activation and increased plasmatic coagulation capacity[115]. Our present findings that abacavir is associated with increased thrombin generation support this hypothesis.

In addition, NRTI-sparing regimens and avoiding zidovudine have been recommended to reduce mitochondrial dysfunction and limit oxidative stress[85,94], which in turn may decrease the risk for cardiovascular disease[86]. Our present findings that $mtDNA_{pl}$ is reduced in PLHIV and that clonal haematopoiesis, a known biomarker for CVD, is linked to reactive oxygen species in PLHIV support the importance of reducing oxidative stress and mitochondrial toxicity by cART modification. This may be achieved by replacing one or both NRTI that are presently the backbone of cART, by antiretroviral drugs from a different class. Currently, multiple studies with dual-drug regimens have shown non-inferiority compared to standard cART regimens for viral suppression[119-121]. Efforts to design and introduce NRTI sparing regimens should be given priority to mitigate NRTI associated toxitiy[94].

Persistent immune activation in thought to be a key factor in the long-term complications of HIV. Thus far, intervention studies with low-dose aspirin (COX-1 inhibition)[100], COX-2 inhibition (NSAIDS)[122,123], sevelamer[124], methotrexate[125,126], mesalamine[127], (hydroxy)chloroquine[128,129], rifaximin[130], valganciclovir[131], dipyramidole[132] showed no or very modest effects on HIV-related inflammation. IL-1β is a key pro-inflammatory cytokine in cardiovascular diseases and malignancies[56,57,133]. Therefore, modulating IL-1β and trained immunity may decrease the risk for cardiovascular disease and cancer in PLHIV[51,54,55,134]. Recently, panobinostat (a HDACi)[44,45], a epigenetic modifier, and canakinumab (IL-1β inhibition)[54] showed potent anti-inflammatory properties in PLHIV. These findings are consistent with our findings in **Chapter 2**. Orally administrated treatments that specifically inhibit the IL-1β pathway, such as inflammasome inhibitors or colchicine may form an attractive alternative to subcutaneous injections[52,58]. However, the actual clinical benefit of the reduction in inflammation was not evaluated in these pilot-studies. In general, studies modulating inflammation in PLHIV with clinically relevant outcome parameters are scarce.

Besides cART modification and possible anti-inflammatory therapies, promoting a healthy lifestyle with a focus on smoking cessation, a healthy diet and reduction in substance-use is key in reducing morbidity in PLHIV[112]. At least 26 behavioural and 34 dietary supplement intervention studies have been registered to reduce HIV-related inflammation on clinicaltrials.gov. These lifestyle interventions should be accompanied by adequate cardiovascular risk management with regard to proper statin prescription and blood pressure control[135]. Reaching adequate LDL levels is important for cardiovascular risk management. Statins have also displayed pleiotropic effects on inflammation[46] and were shown to modulate trained immunity in vitro[136]. Multiple intervention studies have shown beneficial effects of statin on inflammation - independent of cholesterol lowering[46,137-140]. The effect of the statin pitavastatin on clinical outcomes is currently evaluated in a large randomized controlled trial including 6500 PLHIV (REPRIEVE-trial; NCT02344290)[137].

Regarding possible platelet-directed therapies, aspirin is commonly prescribed for secondary prevention of CVD, with $P2Y_{12}$ inhibition therapy for at least one year after myocardial infarction or stent insertion; monotherapy with $P2Y_{12}$ inhibition is common practice after stroke[141,142]. However, long-term $P2Y_{12}$ inhibition (e.g. clopidogrel) instead of aspirin for secondary prevention after myocardial infarction has received growing attention after favourable findings in recent meta-analyses[143,144]. Our finding that low-dose aspirin potentiates innate immune responses (**Chapter 9**), whereas $P2Y_{12}$ inhibition does not or even may reduce inflammation. This supports the prioritization of studies including PLHIV for long-term $P2Y_{12}$ inhibition instead of low-dose aspirin as secondary prevention.

A strength of the current studies was the comprehensive use of a combination of 'multi-omics' technologies with functional immune phenotyping, *ex vivo* validation experiments and the inclusion of validation cohorts. This offers a powerful tool to better understand the underlying biology associated with HIV infection. However, several limitations need to be addressed.

First, even though persistent inflammation has been extensively linked to non-AIDS related co-morbidities, we did not link these disturbances directly to clinical events. While linking these parameters to a clinical outcome is the golden standard, cardiac computed tomography (CT) for calcium scoring, PET imaging for vascular inflammation or intima-media thickness by ultrasound (IMT) could provide clinical intermediate outcomes. Nonetheless, the current set-up is still useful for hypothesis generation. These results could support the rationale for subsequent intervention studies to establish causality and evaluate clinical benefit.

Second, the current PLHIV and control cohort differed in age and sex distribution. Hence, all analyses were corrected for these differences using multivariate analyses with sufficient sample size. Matching on age and sex is often preferred to reduce bias. Still, lifestyle factors, (e.g., smoking and substance-use) and co-medication may influence inflammation and

risk for cardiovascular disease as well. For example, HIV-uninfected men who have sex with men (MSM) showed increased inflammation and microbial translocation compared to non-MSM[145]. Matching controls on all those factors is not feasible. Identifying subgroups and differences within PLHIV and providing sufficient information on those factors can overcome some of these issues and reduces the introduction of bias or overestimation of the effects of HIV itself.

Third, genome-wide association studies in cohorts of PLHIV with large sample sizes have been performed previously[146,147]. Although a 'multi-omics' reduces the required sample size significantly[17,148], the current sample size still limits our discovery rate. Finally, the inclusion of a validation cohort is common practice in genetic studies and is increasingly used in other hypothesis-generating studies.

A larger and/or second multi-omics cohort would tackle most of these limitations. For that reason, a second cohort consisting of PLHIV are enrolled in four centers in the Netherlands (clinicaltrials.gov identifier: NCT03994835). This 2000HIV+ study will contain a discovery and validation cohort, includes sufficient women and will incorporate additional -omics technologies such as transcriptomics and single-cell sequencing. Furthermore, it will include clinical outcome parameters like intima-media thickness and the number and thickness of atherosclerotic plaques in the carotid artery.

In conclusion, HIV care has seen staggering advances over the last decades. Early and effective cART has transformed HIV from a deadly into a chronic condition with a near-normal life expectancy[7,8]. Nonetheless, despite viral suppression there is ongoing immune dysfunction and inflammation, which may be an important target for therapy. In recent years, immune-directed and anti-inflammatory therapies are transforming cardiovascular risk management and treatment of malignancies. I hope the studies described in this thesis add to our understanding of immune and coagulation dysfunction in HIV and, as such, contribute to the identification of effective immune-based therapies for people living with HIV.

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Appendix

Nederlandse samenvatting

Dankwoord/acknowledgements

Research Data Management

Curriculum Vitae

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List of publications

A

Nederlandse samenvatting

Het Humaan immunodeficiëntievirus 1 (HIV-1) werd in 1983 ontdekt als de veroorzaker van het verworven immuundeficiëntiesyndroom (AIDS). Hiv infecteert specifieke afweercellen die de clusterdifferentiatie (CD)4-receptor tot expressie brengen, waaronder T-cellen (CD4+ T-cellen), monocyten/macrofagen en dendritische cellen.

Een primaire hiv-infectie kan 2-4 weken na blootstelling worden waargenomen en presenteert zich meestal als een 'griepachtig' syndroom, hoewel sommige personen geen significante symptomen ervaren. Deze acute hiv-infectie wordt gevolgd door een periode van weinig klinische symptomen (latente fase) of chronische hiv-infectie gedurende 3-20 jaar. Tijdens deze fase nestelt het virus zich in het genetisch materiaal van onze afweercellen. Het virus nestelt zich daarmee levenslang in het menselijk lichaam en wordt het hiv-reservoir genoemd. Zonder behandeling veroorzaakt het natuurlijke verloop van hiv-infectie een daling van CD4+ T-cellen, wat leidt tot ernstige afweerstoornis. Dit leidt tot de ontwikkeling van opportunistische infecties en specifieke vormen van kanker (bv. Kaposisarcoom). Dit syndroom werd AIDS genoemd in de jaren 80. AIDS ging destijds gepaard met een zeer slechte prognose met een levensverwachting tussen 6-12 maanden.

In 1987 werd de eerste antiretrovirale therapie (zidovudine) voor de behandeling van HIV goedgekeurd. Het aanvankelijke gebruik als monotherapie resulteerde in een snelle ontwikkeling van resistentie. Dit belemmerde een succesvolle behandeling op lange termijn. De introductie van meerdere andere antiretrovirale geneesmiddelen in het midden van de jaren negentig maakte de weg vrij voor de introductie van (zeer effectieve) antiretrovirale combinatietherapie (cART), die meestal bestond meestal uit 3 verschillende antiretrovirale middelen. Deze combinatie verhinderde de ontwikkeling van resistentie, waardoor de werkzaamheid van hiv-behandeling opmerkelijk toenam en de prognose van een hiv-infectie sterk verbeterde. Ondanks deze grote vooruitgang hebben mensen met hiv een aanhoudend verhoogd risico op kanker en hart- en vaatziekten met bijbehorend lagere levensverwachting. Dit risico werd in verband gebracht met het laagste bereikte CD4-aantal (CD4-nadir) en reflecteert daarmee ernst van afweerstoornis voor start van behandeling. Dit suggereert blijvende schade bij het uitstellen van de hiv-behandeling. In 2015 toonde een belangrijke hiv-studie aan dat directe start inderdaad de levensverwachting sterk verbeterde. Deze resultaten verenigde de voordelen voor individuele patiënten met voordelen voor de volksgezondheid, aangezien virale onderdrukking als gevolg van antiretrovirale therapie ook het risico op overdracht van HIV tot bijna nul vermindert. Momenteel schat de Wereldgezondheidsorganisatie (WHO) dat 38 miljoen mensen met hiv leven, van wie 25,4 miljoen met antiretrovirale therapie worden behandeld.

Factoren die van invloed zijn op de ontwikkeling van hart- en vaatziekten en kanker bij mensen met hiv zijn complex en omvatten de behandeling zelf, co-infecties (bijv. hepatitis C of CMV-infectie), voedingsstatus, levensstijlfactoren zoals roken en de timing van de start van behandeling. De onderliggende mechanismen zijn onvolledig begrepen. Het risico op deze aandoeningen wordt geassocieerd met ontsteking, onvolledig herstel van de afweer en verhoogde stollingsneiging.

Het menselijk immuunsysteem kan worden onderverdeeld in een aangeboren en een verworven immuunsysteem. Het aangeboren immuunsysteem bestaat uit monocyten, macrofagen, dendritische cellen en bloedplaatjes. De aangeboren immuuncellen reageren niet-specifiek op externe stimuli en produceren ontstekingsmediatoren zoals cytokinen (IL-6, IL-1beta, TNF-alfa) en chemokinen (CCL5, RANTES) die (systemische) ontstekingen veroorzaken. Deze ontstekingsmediatoren zijn in verband gebracht met hart- en vaatziekten, kanker en het risico op overlijden bij mensen met hiv.

Het verworven immuunsysteem bestaat voornamelijk uit T- en B-cellen. Deze cellen zorgen voor een specifieke respons en herbergen een (klassiek) immunologisch geheugen. Ondanks antiretrovirale therapie, is het herstel van CD4+cel aantal na behandeling vaak onvolledig, wat leidt tot langdurige uitputting en veroudering van CD4+cellen. CD4-cellen zijn belangrijk voor de afweer van de darmen en daarom hebben deze veranderingen hebben ook negatieve effecten op de afweer van de darmen. B-cellen zijn belangrijk voor antistofproductie, en ondanks succesvolle behandeling van een hiv-infectie is er aanhoudende B-cel disfunctie en verminderde werking van vaccinaties bij mensen met hiv. Alle compartimenten van het immuunsysteem zijn door al deze verstoringen, ondanks succesvolle onderdrukking van het virus, niet meer in balans. Deze disbalans lijdt tot gezondheidsproblemen bij mensen met hiv.

Het *human functional genomics-project* (HFGP) integreert deze grote hoeveelheid data (-omics) en heeft tot doel ons begrip van de variabiliteit van afweer en ontsteking in de menselijke populatie te begrijpen. Deze aanpak is al succesvol gebleken bij niet-hiv-geïnfecteerde personen. Het leverde nieuwe inzichten op over de invloed van gastheer- en omgevingsfactoren, genetische factoren en darmflora op individuele afweerreacties. Een vergelijkbare benadering kan mogelijk de determinanten van aanhoudende ontsteking bij mensen met hiv ontrafelen en een basis bieden voor nieuwe interventies gericht op het verbeteren van de gezondheid van mensen met hiv. In dit proefschrift bieden we de eerste stappen van deze benadering door veranderingen in stolling, bloedplaatjesfunctie en afweerreactie bij mensen met hiv te onderzoeken. Integratie en koppeling van verschillende (patho)fysiologische processen kunnen de basis leggen voor grotere -omics-gebaseerde projecten die de nieuwe uitdagingen in de zorg en genezing van hiv aanpakken.

In **Hoofdstuk 2** hebben we de aanwezigheid van ontsteking bij mensen met hiv met langdurige virale onderdrukking aangetoond. We hebben daarnaast de functie van circulerende immuuncellen onderzocht. We ontdekten dat stimulatie van circulerende immuuncellen resulteerde in een aanhoudende verhoogde productie van cytokines van monocyten (mn. IL-1β). Deze verhoogde productie is geassocieerd met aanhoudende ontsteking. Dit wijst op een verband tussen veranderingen in de monocyten functie en systemische ontsteking bij mensen met HIV. Deze veranderde functie van monocyten bleek stabiel in de tijd. Monocyten spelen een cruciale rol bij de ontwikkeling van atherosclerose (aderverkalking). Deze cellen kunnen na blootstelling aan endogene en microbiële structuren opnieuw worden geprogrammeerd door veranderingen aan te brengen op epigenetisch (veranderingen aan de bereikbaarheid van het DNA) niveau. Dit proces wordt 'getrainde afweer of immuniteit' genoemd. Het getrainde fenotype maakt monocyten met een langdurige verbeterde functionele toestand met een versterkte afweerreactie (bijv. IL-6- en IL-1β-productie) op een tweede stimulus. Een bekende veroorzaker van deze getrainde afweer is β -glucan, een onderdeel van schimmels. We vonden dat β -glucan verhoogd was bij mensen met HIV en met name in de groep met meest pro-inflammatoire monocytfunctie. Klinische consequenties van getrainde afweer zijn aangetoond bij meerdere ziekten zoals hart- en vaatziekten en diabetes. Onze gegevens wijzen op een vergelijkbaar getraind afweer in mensen met hiv.

In **Hoofdstuk 3** hebben we ons gericht op de samenstelling van de circulerende afweercellen door 108 verschillende celtypes bij mensen met hiv te onderzoeken en deze te vergelijken met gezonde controles. Ondanks langdurig antiretrovirale therapie, vertoonde mensen met hiv significante verandering in de verhouding van verschillende immuuncellen. De verhouding tussen pro-inflammatoire Th17-cellen (een specifieke CD4 T-cel) en onderdrukkende regulatoire T-cellen (Treg) moet in evenwicht blijven om functionele afweer te behouden. Veranderde verhoudingen zijn beschreven bij onbehandelde hiv-infectie (lagere Th17/Treg), auto-immuunziekte en kanker (hogere Th17//Treg).

Deze veranderingen in immuuncel aantallen vertaalden zich ook in functionele veranderingen in de verworven afweer met verminderde cytokineproductie door T-cellen. Parameters van het hiv-reservoir correleerden met deze veranderingen en met verschillende circulerende afweercellen. Naast het hiv-reservoir droegen ook CMV-co-infectie (virus), ernst van ziekte bij start van behandeling, leeftijd, geslacht en roken bij aan de samenstelling van afweercellen in ons menselijk lichaam. Deze verstoringen kunnen een rol spelen bij het in stand houden van het hiv-reservoir. Vervolgonderzoek zal zich focussen op het moduleren van deze veranderingen om hiv-genezing dichterbij te brengen.

In **Hoofdstuk 4** rapporteren wij het optreden van somatische mutaties in ons DNA die leiden tot klonale hematopoëse in mensen met hiv. Hematopoëse is de productie van bloedcellen. Klonale hematopoëse is de aanwezigheid van een kloon van gemuteerde bloedcellen en komt voor bij gezonde individuen uit de algemene populatie. Het risico op een kloon neemt toe met de leeftijd. Deze klonen zijn geassocieerd met het risico op hart- en vaatziekten en hematologische maligniteiten in de algemene populatie. Wij toonden aan dat mensen met hiv meer klonale hematopoëse hebben en dat dit geassocieerd is met ernst van eerdere infectie (laagste CD4-getal), hiv-reservoir en leeftijd. Daarnaast was klonale hematopoëse ook geassocieerd met stollingsactiviteit in mensen met hiv.

Eerdere studies hebben aangetoond dat D-dimeer concentraties verhoogd zijn bij mensen met hiv en verband houden met mortaliteit en het optreden van hart- en vaatziekten. D-dimeer is een biomarker van stolling. Tot op heden zijn de gepubliceerde gegevens over de stollingscapaciteit bij mensen met hiv tegenstrijdig. Het vermogen van plasma om trombine te vormen is een belangrijke factor in stollingscascade. Trombine productie kan worden gemeten in een gestandaardiseerde setting. In **Hoofdstuk 5** beschrijven wij trombine productie in ons 200HIV-cohort en toonden wij aan dat het gebruik van abacavir (een antiretroviraal middel) onafhankelijk geassocieerd was met een verhoogde stollingscapaciteit. Abacavir is in het verleden gelinkt aan hart- en vaatziekten.

Bloedplaatjes spelen naast plasmafactoren ook een belangrijke rol in stolling en wondgenezing. Naast deze rol, spelen zij ook een belangrijke rol bij ontsteking en ontwikkeling van hart- en vaatziekten. Bloedplaatjes hebben geen kern maar beschikken over mitochondriaal DNA (mtDNA). Mitochondriën zijn belangrijk voor de energiehuishouding van alle cellen in ons lichaam. Mitochondriële toxiciteit is een belangrijk onderliggend mechanisme voor het optreden van de belangrijkste bijwerkingen bij bepaalde groep van antiretrovirale middelen (NRTI). In **Hoofdstuk 6** laten wij zien dat bloedplaatjes van mensen met hiv minder mtDNA bevatten. Deze verlaging is het meest uitgesproken bij mensen dien in het verleden zidovudine gebruikt hebben. Zidovudine is één van de eerste antiretrovirale middelen en is sterk gelinkt aan mitochondriële toxiciteit. Deze mitochondriële disfunctie leidt in bloedplaatjes tot een lager energieniveau en zorgt daarmee ook voor verminderde functie van bloedplaatjes. Mitochondriële disfunctie is geassocieerd met ontsteking.

In **Hoofdstuk 7-9** beschrijven we aanvullende studies om de mechanismen die bloedplaatjesfunctie koppelen aan ontsteking, afweer en antiretrovirale medicatie te onderzoeken. Een eerdere studie van onze groep suggereerde dat personen met een op raltegravir gebaseerd regime een verminderde bloedplaatjesfunctie hadden in vergelijking met personen met een ander antiretroviraal regime.

Hoofdstuk 7 rapporteert de resultaten van een gerandomiseerde gecontroleerde studie waarin de effecten zijn onderzocht van het overschakelen van hiv-behandeling naar een regime gebaseerd op de integraseremmer raltegravir. We laten zien dat het overschakelen naar raltegravir gedurende tien weken de bloedplaatjesfunctie niet veranderde.

Bloedplaatjes moduleren ons immuunsysteem door direct contact met andere andere immuuncellen. In **Hoofdstuk 8** hebben we het effect van bloedplaatjes op regulerende T-cellen (Treg) onderzocht. Deze anti-inflammatoire cellen kunnen ook pro-inflammatoire kenmerken ontwikkelen. Bloedplaatjes kunnen deze ontwikkeling remmen en voorkomen dat deze pro-inflammatoire eigenschappen ontwikkelen. Bloedplaatjes van mensen met hiv kunnen minder goed binden aan Tregs, wat zou kunnen leiden T-cellen die meer ontstekingsmediatoren produceren zoals IFN-gamma en IL-17.

Er zijn verschillende geneesmiddelen gebruikt om de bloedplaatjesfunctie te remmen en daarmee hart- en vaatziekten te voorkomen. Een lage dosis acetylsalicylzuur (ASA of aspirine) wordt traditioneel gebruikt voor de remming van bloedplaatjes. Aspirine wordt steeds vaker gecombineerd met of vervangen door P2Y12-remmers, zoals clopidogrel en ticagrelor. In **Hoofdstuk 9** hebben we de effecten geëvalueerd van aspirine, ticagrelor en clopidogrel op ontsteking tijdens menselijke endotoxemie, een bekend model om ontsteking bij mensen te bestuderen. In dit model wordt in gezonde vrijwilligers een onderdeel van de celwand van een bacterie ingespoten waarbij er een (kortdurende) ontstekingsreactie ontstaat, vergelijkbaar met een bloedvergiftiging. Hoewel het effect van P2Y12-remming op ontsteking beperkt was, versterkte een zevendaagse kuur met een lage dosis aspirine de ontstekingsreactie (bijv. TNFα), hoogstwaarschijnlijk door het remmen van een enzym (COX-1) in bloedplaatjes. Aspirine staat algemeen bekend om zijn ontstekingsremmende eigenschappen en is daarom gebruikt voor de behandeling van aanhoudende hiv-gerelateerde ontstekingen, zij het met beperkt succes. Onze gegevens bieden een verklaring waarom een lage dosis aspirine in eerdere onderzoeken niet resulteerde in een detecteerbaar ontstekingsremmend effect en mogelijk zelfs de ontsteking bij ontstekingsziekten verhoogt.

Het ontwikkelen van gerichte behandeling voor aanhoudende ontsteking en afweerdisfunctie in mensen met hiv kan de zorg voor deze mensen sterk verbeteren. De volgende stap is het extrapoleren van onze resultaten naar de ontwikkeling van nieuwe behandelstrategieën. Vooralsnog is de belangrijkste stap de vroege start van antiretrovirale therapie, waardoor het grootste deel van de verhoogde mortaliteit geassocieerd met hiv wordt verminderd. Toch zal de laatste stap om de levensverwachting van mensen met hiv volledig te normaliseren ook gerichte therapie op immuundisfunctie moeten worden gericht.

Interventiestudies met meerdere ontstekingsremmers, bloedverdunners, cholesterol-verlagers of antivirale medicijnen lieten geen of zeer bescheiden effecten op hiv-gerelateerde ontsteking zien. IL-1β is een belangrijke pro-inflammatoire cytokine bij hart- en vaatziekten en maligniteiten. Daarom kan het moduleren van IL-1β en getrainde immuniteit het risico op hart- en vaatziekten en kanker bij mensen met hiv mogelijk verminderen. Onlangs vertoonden panobinostat, een epigenetische modulator, en canakinumab (IL-1β-remming) krachtige ontstekingsremmende eigenschappen bij mensen met HIV. Deze bevindingen komen overeen met onze bevindingen in **Hoofdstuk 2**. Oraal toegediende behandelingen die specifiek de IL-1β-route remmen, zoals anti-jicht medicijn colchicine, kunnen een aantrekkelijk alternatief vormen voor injecties. Het werkelijke klinische voordeel van de vermindering van ontsteking werd echter niet geëvalueerd in deze relatief kleine studies. Grote studies zijn hiervoor in de toekomst nodig. Het bevorderen van een gezonde levensstijl met een focus op stoppen met roken, een gezond dieet en vermindering van het middelengebruik zijn verder ook van cruciaal belang bij het verminderen van de morbiditeit bij mensen met hiv.

De kracht van uitgebreide karakterisering van de afweer en ontstekingsreactie van mensen met hiv heeft in dit proefschrift geleidt tot nieuwe inzichten. Een vervolgstudie met 2000 deelnemers is reeds gestart om onze bevindingen te bevestigen en nieuwe analyses toe te voegen met als doel om nieuwe invalshoeken te vinden ter verbetering van de zorg voor mensen met hiv. Dit tweede cohort, bestaande uit mensen met hiv ingeschreven in vier centra in Nederland, zal ons begrip van hiv verder verbeteren.

Samenvattend: de hiv-zorg heeft de afgelopen decennia enorme vooruitgang geboekt. Vroege en effectieve antiretrovirale therapie heeft hiv getransformeerd van een dodelijke ziekte naar een chronische aandoening met een bijna normale levensverwachting. Desalniettemin is er, ondanks virale onderdrukking, aanhoudende ontsteking en bijbehorende veranderingen in de afweer. Deze afwijkingen kunnen een belangrijk doelwit voor therapie zijn en daarmee ziekten zoals kanker en hart en vaatziekten voorkomen. In de afgelopen jaren hebben deze ontstekingsremmende therapieën het cardiovasculaire risico en de behandeling van maligniteiten getransformeerd. Ik hoop dat de studies die in dit proefschrift worden beschreven, bijdragen aan ons begrip van afweer en stollingsdisfunctie bij mensen met hiv en als zodanig bijdragen aan de identificatie van effectieve immuungebaseerde therapieën voor mensen die leven met hiv.

Acknowledgements - Dankwoord

Velen hebben bijgedragen aan de totstandkoming van dit proefschrift. Graag wil ik aantal mensen in het bijzonder bedanken.

Beste **Andre**, mijn dank is groot en niet goed uit te drukken in dit dankwoord. Je energie en passie voor wetenschap werken aanstekelijk. Je creativiteit en betrokkenheid zijn inspirerend. De brede interesse weet je ook nog eens om te zetten in grote onderzoeksprojecten (sysmex en 2000HIV). Het was mooi om dit van dichtbij mee te maken. Maar ook je strenge woorden waren belangrijk voor de totstandkoming van dit werk. Je onmetelijke ondersteuning en laagdrempelige bereikbaarheid waren erg prettig (en onmisbaar) tijdens de 'eindsprint) van mijn proefschrift.

Beste **Mihai**, dank voor de samenwerking. Je creativiteit, analytisch vermogen en efficiëntie zijn indrukwekkend. Het is echt een privilege geweest om dat van dichtbij mee te maken. Ondanks je erg drukke agenda was er altijd tijd voor een kort overleg en was de deur open voor vragen.

Q, ik dank mijn beurs en promotieplek aan je initiele vertrouwen en ondersteuning bij het schrijven van een PhD beurs. Jouw enthousiasme voor het vak van (tropische) infectieziekten heeft me vaak (bijna) overgehaald om toch infectieziekten te willen doen. Dank voor de prettige samenwerking. Ik heb veel geleerd van je kritisch benadering bij het schrijven van de verschillende artikelen. Je was een belangrijk onderdeel van dit proefschrift.

Lisa, het was een mooie tijd. Ik heb ongelofelijk veel bewondering voor jouw werklust, enthousiasme en doorzettingsvermogen. De gezelligheid en slimme sparring partner heb ik als zeer waardevol ervaren. Je inzet was altijd 100% en dit wist je te combineren met een indrukwekkend druk sociaal leven. Je staat altijd klaar voor anderen en dat is inspirerend. Ik hoop in de toekomst nog veel te mogen samenwerken. Floor, jouw doorzettingsvermogen en efficiëntie zijn bewonderingswaardig. Je bent een perfecte collega. Onze samenwerking smaakt naar meer.

Dear **Charles**, it has been a pleasure to work with you. Your knowledge and input have been instrumental to this thesis. Thank you for the support and discussions.

Beste **Philip** en **Mark**, dank voor jullie onuitputtelijke plaatjes kennis. Jullie kritisch blik en brainstormsessies zijn een belangrijk onderdeel van dit proefschrift.

Martin, je niets aflatende positieve instelling en (lab)ondersteuning hebben mij ontzettend geholpen. Je engelen geduld met weer zo'n dokter die denkt dat hij kan pipeteren is bewonderenswaardig. Echt een voorbeeld van de ongelofelijk prettige sfeer binnen het lab en onderzoeksgroep. Jouw fanatieke (duitse) gezelligheid bij elke borrel heb ik ook erg gewaardeerd. Rob, je hulp tijdens (weer) een nieuwe R-crisis was onontbeerlijk. Dank voor de fijne samenwerking en gezelligheid. Dank ook voor je niet aflatende energie en inzet bij het Lowlandsproject. Een mooie herinnering aan dit promotietraject.

Alexander, **Rosanne** en **Marloes**, de samenwerking bij het laatste hoofdstuk heb ik als heel erg prettig ervaren. Een voorbeeld voor toekomstige projecten.

Ajeng, **Vesla**, **Godfrey**,**Nadira**, **Berenger**, **Mike**, **Bony**, **SIlvita**, **Annelies**, **Fadel**, **Hinta**, **Khutso**, your support, 'gezellligheid' and interesting discussions have been a real treat. It has made this journey all the more enjoyable. Thank you for everything.

Marjolein, **Bert** en **Karin**, jullie ondersteuning voor de verschillende studies zijn erg belangrijk geweest in de totstandkoming van dit proefschrift. De positieve woorden van de meeste deelnemers over jullie zegt genoeg. Dank voor de inzet naast jullie andere werkzaamheden.

Dorien, **Matthijs**, **Guus** en **Peter**, dank voor de prettige samenwerking. Ik kijk uit naar de volgende fase in onze samenwerking.

Beste **Linos**, **Sofie** en **Wim**, jullie kennis over het hiv-reservoir hebben een belangrijke rol gespeeld in het succes van het 200HIV project. Ik hoop dat het een blijvende samenwerking zal worden.

Beste **Sip**, **Hans**, **Irma**, **Bram** en **Esther**, de gastvrijheid en ondersteuning op jullie lab waren erg prettig. De samenwerking bij 1 van mijn eerste projecten was echt een warm bad.

Alle **collega's van het EIG** en in het bijzonder; **Cor, Liesbeth, Heidi, Helga, Kiki, Trees, Sanne, Marije, Arjan, Rob, Michelle, Maartje, Intan, Siroon, Bas, Lazlo, Mark, Jaap, Ekta, Jessica, Janna, Valerie, Ruud, Yvette, Inge, Charlotte, Sam, Jorge, Anne, Berenice, Inge, Freek, Hedwig, Laszlo, Rinke, Lily, Mariolina, Mariska, Marlies, Tania, Viola, Nico, Simone, Vera, Jacqueline, Duby, Thijs, Dennis, Hanne, Rutger, Leo** and **Reinout** voor de samenwerking, gezelligheid en geduld als er 'weer' een dokter met een pipet aan de slag gaat in het lab. Het was mij een waar genoegen.

I would like to thank Viiv for their belief in the Human Functional genomics project and their commitment to a very interesting new chapter to HIV research at our institution (2000HIV project). Their support has also improved the final papers of this thesis. I would like to wish the new team (Louise, Maartje, Marc, Willem, Jessica and others) success with this interesting project.

Collega's in het JBZ, dank voor de samenwerking. Jullie gezelligheid maakte de combinatie van kliniek en onderzoek dragelijk. **Watske** en **Paetrick** dank voor jullie ondersteuning en flexibiliteit ten aanzien van mijn combinatie van onderzoek en opleiding. Zonder jullie steun en geduld was dit proefschrift niet tot stand gekomen.

Lieve **vrienden**, dank voor alle gezelligheid, afleiding en vriendschap. De etentjes, tenniswedstrijden, reizen, karaokesessies (It's all coming back to me now), verre reizen, lange terras-sessies, felle discussies, slap gelul en eindeloze koffiedates zijn intrinsiek verbonden aan dit werk en daarom onmisbaar in dit dankwoord. De interesse in de voortgang werd gewaardeerd ondanks mijn soms korte respons; 'laten we het gezellig houden'. Ik kijk uit wat onze vriendschap nog meer gaat brengen.

Lieve **familie**, dank jullie wel voor jullie begrip, interesse, afleiding en ondersteuning. Jullie bijdrage heeft dit proefschrift mogelijk gemaakt. Ik prijs mij gelukkig.

Lieve **Evelien**, onderzoek is zeg maar niet helemaal jouw ding. En mijn PhD al helemaal niet. Ik ben ongelofelijk blij met je. Jouw ondersteuning en begrip hebben een grote rol gespeeld in het afronden van dit proefschrift. Ik kijk met grote bewondering naar jouw efficiëntie om werk, privé en neventaken te combineren. 'Goed voorgaan, doet goed volgen'. Ik kijk uit naar onze komende avonturen samen, **Lieve** en …

Research Data Management

The data obtained for this thesis are stored according to the FAIR principles to optimize Findability, Accessibility, Interoperability and Reusability[1]. Raw and processed data for Chapter 2-7 were stored digitally on a local server of the department of Internal Medicine (H:)\\umcfs073\aigdata$\ExpIntGnk\500FG_project, which is backed-up daily on the Radboudumc server. Accessibility was limited to researchers directly involved in the 200HIV/500FG projects within the human functional genomics project (HFGP). For Chapter 8 and 9, data of participants were stored (H:)\\umcfs073\aigdata$\ExpIntGnk\Employees\ WoutervanderHeijden\RAPID. Data for chapter 10 were stored (H:)\\umcfs073\aigdata$\ ExpIntGnk\Employees\ WoutervanderHeijden\TICA. All human studies described in this thesis were conducted according to the principles of the declaration of Helsinki and were approved by the Medical Ethics Committee on Research Involving Human Subjects, region Arnhem Nijmegen, Nijmegen, The Netherlands. Prior to enrolment all participants gave written informed consent. In chapter 2-7, participants received electronic questionnaires via Castor EDC (Amsterdam, The Netherlands). Data management and monitoring were performed within Castor EDC for all studies involving human subjects. The data will be saved for 15 years after termination of the study as per informed consent in case of Chapter 2-7 (February 2032), Chapter 8-9 (November 2029) and Chapter 10 (November 2030). An independent Data Safety Monitoring Board was (DSMB) was established for the study described in Chapter 10 to perform safety surveillance and interim analyses on safety data halfway through the study. Using these patient data in future research is only possible within the framework of the Human functional genomics project (www.humanfunctionalgenomicsproject.org). If additional questions arise a renewed informed consent should be obtained as recorded in the informed consent. Digital copies of the informed consent forms are stored at (H:)\\umcfs073\aigdata$\ExpIntGnk\ 500FG_project. Hard copies are stored in a locked closet (M379.01.152) to which only Wouter van der Heijden and Lisa Van de Wijer have access. The handling of subject data in all studies complied with the Dutch Personal Data Protection Act (in Dutch: De Wet Bescherming Persoonsgegevens, Wbp). The privacy of the participants was warranted by use of encrypted and unique individual subject codes. The coding information is stored digitally separately from data and informed consent forms which has been secured with a password known by Wouter van der Heijden and Lisa Van de Wijer. Data generated in this thesis are part of published articles. RNAseq data described in chapter 2 have been deposited in the public functional genomics data repository GEO database with accession number GSE160184. Immunological data (immunophenotyping and cytokine production) and neuropsychiatric symptom questionnaire data are deposited at the DANS EASY archive of the Radboud university which is certified with the CoreTrustSeal, identifier is available upon request from Wouter van der Heijden or Lisa Van de Wijer. and files are available from the corresponding author upon reasonable request. All left-over samples are stored at M379.01; freezer 9; rack 9 at -80 degrees Celsius (with exact lay-out in folder: H:)\\umcfs073\aigdata$\ExpIntGnk\general\10 storage databases\Freezers. Furthermore, samples of the HFGP cohort are also stored at the Radboud biobank and available upon reasonable request. Data for Chapters 2-6 were analysed using R 3.6. Data from Chapters 7-10 were analysed with SPSS Version 22 (SPSS Inc.), Graphpad Prism 5 or Kaluza Software (Beckman-Coulter).

1 Wilkinson MD, Dumontier M, Aalbersberg IJ, Appleton G, Axton M, Baak A et al. The FAIR Guiding Principles for scientific data management and stewardship. *Sci Data 3, 160018, doi:10.1038/sdata.2016.18 (2016).*

Curriculum Vitae

Wouter van der Heijden werd op 15 oktober 1987 geboren in Utrecht en groeide op in De Meern samen met zijn broer en zus. In 2005 behaalde hij zijn VWO diploma aan het Leidsche Rijn college te Utrecht, na een jaar International Economics in Groningen, begon hij aan de studie Geneeskunde aan de Radboud universiteit te Nijmegen. Tijdens zijn opleiding liep hij stage in Moshi, Tanzania en Cuenca, Ecuador. Hij nam deel aan het Honours Programma van de Radboud Universiteit. Hij deed zijn eerste onderzoekservaring op bij Prof. Andre van der Ven over niertoxiciteit van HIV medicijnen. Na het behalen van het artsexamen in 2013 begon hij als ANIOS interne geneeskunde in het Gelderse Vallei ziekenhuis in Ede, om vervolgens in 2014 te starten met de opleiding tot internist in het Jeroen Bosch Ziekenhuis te Den Bosch onder begeleiding van dr. Watske Smit. Dit combineerde hij vanaf oktober 2014 met een promotietraject in het Radboudumc onder begeleiding van prof. dr. André van der Ven en dr. Quirijn de Mast. Daarbij onderzocht hij de immunologische gevolgen van een chronische HIV-infectie met een speciale interesse voor bloedplaatjes en monocyten. In 2021 vervolgde hij zijn opleiding in het Radboudumc onder begeleiding van Prof. dr. Jacqueline de Graaf en later dr. Gerald Vervoort. Per 2022 zal Wouter zich toespitsen op de Intensive Care geneeskunde binnen het Radboudumc als Fellow Intensive Care. Wouter woont samen met zijn vriendin Evelien van Eeten en hun dochter Lieve in Beek-Ubbergen.

PhD Portfolio

Name PhD candidate: W.A. van der Heijden
Department: Internal Medicine
Graduate School: RIHS

PhD period: 01-10-2014 – 31-12-2020
Promotor(s): Prof. A.J.A.M. van der Ven
Prof. dr. M.G. Netea
Co-promotor(s): dr. Q. de mast

	Year(s)	ECTS
TRAINING ACTIVITIES		
Courses & Workshops		
Radboudumc Introduction day	2014	0.5
RIHS specific introductory course	2015	1.0
BROK course	2015	2.0
Scientific integrity course	2016	1.0
Basic course in R, Erasmus MC	2016	2.5
BROK cursus (NFU)	2016	2.0
Academic writing course	2017	2.0
Post-CROI Conference	2019	0.25
Seminars & lectures		
Radboud Research Rounds (monthly)	2015-2019	1.0
Cytokine meetings Internal Medicine (weekly; incl. 5 x oral)	2015-2019	4
NVHB meeting (oral)	2017	0.5
Symposia & congresses		
EACS 2013, Brussels (Oral)	2013	1.0
Radboud Science Day Infectious Diseases (laptop)	2015	0.5
Retraite AMC-Radboudumc (laptop)	2016	1
International Platelet meeting 2016, Wellesley (Oral and Poster)	2016	2
ISLH conference, Milan (Oral)	2016	1.5
Summer Frontiers - Systems Biology of Innate Immunity (poster)	2016	1.5
New Frontiers Symposium on the Microbiome , Nijmegen	2017	1.0
Retraite AMC-Radboudumc (laptop)	2017	1
CROI Conference 2017, Seattle (Poster)	2017	2
Radboud Science Day Infectious Diseases (oral)	2018	0.5
Frontiers in Innate Immunity and Inflammation (Poster), Cluj	2018	1.5
AIDS Conference 2020, San Francisco digital (poster)	2020	2

TEACHING ACTIVITIES		
Lecturing		
Lecturer in bachelor course Severe Infectious Diseases	2015-2018	1.5
Lecturer in minor Global Health & Infectious Diseases	2017	0.5
Supervision of internships / other		
Supervision Medical student (total 3 months)	2016	1.0
Supervision Biomedical sciences student (total 6 months)	2016	2.0
Supervision Biomedical sciences student (total 6 months)	2017	2.0
TOTAL		**39.25**

List of publications

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